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Arbuscular mycorrhiza in *Medicago truncatula*: Involvement of *MtHA1* in formation and symbiotic functions and molecular analysis of interaction with a root pathogen

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There is only one heroism in the world：

to see the world as it is and to love it.

Romain Rolland

Abstract

Arbuscular mycorrhiza(AM) is a wide spread mutualistic symbiosis, which can improve phosphate acquisition and pathogen resistance of plants. In the current Ph.D. thesis the role of a proton pumping ATPase(MtHA1) for the AM symbiosis in*Medicago truncatula*was investigated. In *MtHA1*mutant plants, differentAM fungi only developed truncated arbuscules without forming typical hyphal branches, and thisphenotype was mirrored by expression patterns of genes for proteins located in different areasof the periarbuscular membrane. AM fungal colonization, improved phosphate uptake and plant growth promotion werereduced in *MtHA1*mutant plants. Mycorrhiza-induced resistance (MIR) and the nodule symbiosis were, however, not affected.

MIR was further analyzed in the *M. truncatula*infected with *Aphanomyces euteiches*which causesa root-rot disease in legumes. Ina split root system showing high levelsof defense-gene expression, colonization of an AM fungus reduced this expression and inconsequence increased susceptibility of the roots for the pathogen. In roots of pot cultures, however, a typical MIR was observed and could be based on the higher activity of jasmonate/ethylene-regulated defense responses due to suppression of salicylic acid biosynthesis.

In conclusion, this work shows that the gene *MtHA1*encoding a proton pumping-ATPase plays a critical role in the formation and function of arbuscule-containing cells. Expression of the mutated gene results in reduced formation of arbuscule branches. This in turn negatively influences mycorrhizal phosphate uptake, plant growth promotion and overall mycorrhizal colonization of the roots. Gene expression analyses indicate that different mechanisms underlay local and systemic interactions between the mycorrhizal fungus and the root pathogen.The different physiological stages of pot culture and split root system make a comparison of the two experimental approaches, however, difficult.

Keywords:*Medicago truncatula*, proton pump ATPase, arbuscular branches, phosphate uptake, mycorrhiza-induced resistance, defense-related gene expression, split-root system

Zusammenfassung

Die arbuskuläre Mykorrhiza (AM) ist eine mutualistische Symbiose, die die Phosphataufnahme und Pathogenresistenz von Pflanzen verbessern kann. In der vorliegenden Doktorarbeit wurde die Rolle der Protonen-pumpenden ATPase MtHA1 für die AM Symbiose in *Medicago truncatula* untersucht. In *MtHA1* Mutanten konnten AM Pilze nur noch verkürzte Arbuskel ohne typische Verzweigungen ausbilden. Dies zeigte sich auch in Expressionsmustern von Genen, die für Proteine in verschiedenen Bereichen der periarbuskulären Membran kodieren. Außerdem waren AM Pilzbesiedelung, die verbesserte Nährstoffaufnahme und die Wachstumsförderung in MtHA1 mutierten Pflanzen reduziert.

Die Mykorrhiza-induzierte Resistenz (MIR) wurde näher in *M. truncatula* Pflanzen untersucht, die von *Aphanomyces euteiches* infiziert waren, dem Erreger einer Wurzelfäule in Leguminosen. In einem geteilten Wurzelsystem, das eine hohe Expression von Verteidigungsgenen aufwies, unterdrückte ein AM Pilz diese Expression und erhöhte in Folge die Empfindlichkeit für das Pathogen. In Wurzeln von Topfkulturen dagegen konnte eine typische MIR beobachtet werden, die wahrscheinlich auf erhöhter Aktivität der Jasmonat/Ethylen-regulierten Verteidigungsantwort beruht, verursacht durch eine Unterdrückung der Salizylsäuresynthese.

Im Ergebnis zeigt diese Arbeit die bedeutende Rolle des Gens *MtHA1* für die Bildung und Funktion der arbuskelhaltigen Zellen. Die Mutation des Gens führt zur verminderten Arbuskelverzweigung, reduzierter Phosphataufnahme und Wachstumsförderung in der Mykorrhiza und schließlich zu einer geringeren Gesamtbesiedelung durch den AM Pilz. Genexpressionsanalysen weisen darauf hin, dass unterschiedliche Mechanismen den lokalen und systemischen Wechselwirkungen zwischen AM Pilzen und Pathogenen in der Wurzel zu Grunde liegen. Verschieden physiologische Zustände von geteilten Wurzelsystemen und Wurzeln in Topfkulturen erschweren allerdings einen direkten Vergleich der beiden experimentellen Ansätze.

Stichworte: *Medicago truncatula*, Protonen-pumpenden ATPase, Arbuskel-Verzweigungen, Phosphataufnahme, Mykorrhiza-induzierte Resistenz, Expression von Verteidigungsgenen, geteilte Wurzelsysteme

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Abbreviations

ABA	Abscisic Acid
AM	Arbuscular Mycorrhizas
AMS	Arbuscular Mycorrhizal Symbiosis
ANOVA	Analysis of variance
DW	Dry Weight
ET	Ethylene
FW	Fresh Weight
HET	Heterozygote
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
MDG	Mycorrhizal Growth Dependency
MIR	Mycorrhiza-Induced Resistance
MRI	Mycorrhiza shoot/root Ratio (MRI) Increase
MUT	Mutant
P	phosphorus
P_i	inorganic phosphate
PPA	Pre-penetration apparatus
PR	Pathogenesis-related proteins
RNS	Root Nodule Symbiosis
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
v/v	volume per volume
WAI	Weeks After Inoculation
WT	Wild type

1 General introduction

1.1 General introductionto arbuscular mycorrhiza

Mycorrhizas are mutualistic associations formed between plant roots and fungi. The symbiosis is named after the Greek words "*mycos*" and "*rhiza*" meaning "fungus-root" (Smith and Read, 2008). Based on the behavior of the fungal partner to penetrate individual root cells or not, mycorrhizas can be divided into two types, ectomycorrhizas and endomycorrhizas. Ectomycorrhizal fungi are characterized by formation of a mantle or sheath surrounding the root and intercellular hyphae growing between the cells of the root cortex forming the so-called Hartig net (Smith and Read, 2008). Ectomycorrhizas can be formed between approximately 10% of all plant families and the fungi from the phyla Basidiomycota and Ascomycota and occasionally Zygomycota (Tedersoo et al., 2010). Ectomycorrhizas are distributed throughout boreal, temperate and tropical ecosystems, and play important roles such as improving mineral nutrient acquirement (mainly nitrogen), reducing metal ion toxicity, and improving tree water economy (Peay et al., 2007; Smith and Read, 2008). Endomycorrhizas are further subdivided into arbuscular mycorrhizas, ericoid mycorrhizas, arbutoid mycorrhizas, monotropoid mycorrhizas, ectendomycorrhizas and orchid mycorrhizas and, this classification is mainly based on profound differences of intracellular hyphal development in the apoplast after penetration of the cell wall (Peterson et al., 2004; Smith and Read, 2008).

Arbuscular mycorrhiza (AM) is the most wide-spread form of mycorrhizal associations and of great ecological and economic importance (Francis and Read, 1994; Jeffries et al., 2003; Fitter, 2005; Smith et al., 2009) The symbiosis is formed between fungi of the monophyletic phylum Glomeromycotaand more than 80% of all terrestrial plant species, including angiosperms, gymnosperms, pteridophytes, lycopods, and mosses (Schüßler et al., 2001). Only a few plant species like members of the Brassicaceae, Caryophyllaceae, Chenopodiaceae, or Urticaceae cannot form AM (Wang and Qiu, 2006). The AM is an ancient symbiosis that can be traced back to more than 450 million years ago based on fossil records and phylogenetic evidences (Remy et al., 1994; Taylor et al., 1995; Redecker et al., 2000; Brundrett, 2002; Wang and Qiu, 2006). At that time plants started to colonize the land implying that the AM symbiosis was functional in this process and that it presents the mother of different kinds of root symbioses (Brundrett, 2002; Parniske, 2008).

Observation of mycorrhizal structures inside roots was documented more than one hundred years ago (Frank, 1885), but early taxonomic classification of AM fungi almost entirely relied on the development and wall structure of the spores (Walker, 1992). The first fungus which was identified to form mycorrhizal structures was later described as *Glomus mosseae* (Mosse, 1953). Based on morphological similarity, the AM fungi were initially grouped in one genus *Endogone* in a single family Endogonaceae (Zygomycota) (Thaxter, 1922). With the establishment of two other genera *Acaulospora* and *Gigaspora*, the genus *Glomus* was then separated from the genus *Endogone* but still in thefamily Endogonaceae (Gerdemann and Trappe, 1974). In a new classification system, a new order (Glomerales) was proposed by Morton and Benny (1990) to include all AM fungi in one phylogenetic group (Glomales) and it consisted of two suborders (Glomineae and Gigasporineae) and three families (Glomaceae, Acaulosporaceae and Gigasporaceae). Phylogenetic analyses of small subunit (SSU) rRNA gene sequences at the start of the 21st century separated AM fungi from the polyphyletic Zygomycota and placed them into a new monophyletic phylum, the Glomeromycota (Schüßler et al., 2001). As the SSU sequence is too conserved to resolve species level in Glomeromycota, the current classification (www.amf-phylogeny.com) was set up based on the sequences of the large subunit (LSU) rRNA gene, the internal transcribed spacer (ITS) region and the gene encoding β-tubulin (Schüßler and Walker, 2010). In this newest classification, new genera, such as *Funneliformis* which have the funnel-shaped spore base and a unique SSU sequence, and *Rhizophagus* which form abundant spores in root of vascular plants andgenetically falls into the same clade with *Rhizophagus populinus*,were set up in the family Glomeraceae (Dangeard, 1900; Gerdemann and Trappe, 1974; Schüßler and Walker, 2010).

1.2 Development of arbuscular mycorrhizas

As obligate biotrophs, most processes of arbuscular mycorrhizal fungal development depend on the host plant: This comprises three stages: germination of propagules, accommodation of AM fungi, and extension of intra- and extra-radical hyphae with formation of new propagules (Smith and Read, 2008).

1.2.1 Germination of propagules

Different forms of propagules (spores, root fragments, and hyphal network) from AM fungi exist in natural environments (Tommerup and Abbott, 1981; Friese and Allen, 1993; Merryweather and Fitter; 1998; Klironomos and Hart, 2002). Initially used in AM fungal

classification, spores are probably the most studied propagules due to the early developed 'wet-sieve' method (Gerdemann and Nicolson, 1963). In natural environment, spore density and composition vary based on sampling season, habitat and host plant. In frequently disturbed agricultural soil, the spore number of the genus *Glomus* increases while spore numbers of other genera such as *Gigaspora* are reduced because they require longer time for sporulation (Franken and George, 2006). There are three different ways how AM fungal spores germinate: through the germination shield, through the spore wall or through the hyphal attachment (Siqueira et al., 1985). Due to the lack of carbon uptake and lipid biosynthesis (Pfeffer et al., 1999), germinated AM fungal spores can support hyphal development only for a limited time (asymbiotic development). During this time, the main carbon storage compounds (triacylglycerides and glycogen) of the fungus are used to develop the coenocytic germ tubes and provide carbon skeletons for anabolism (Bago et al., 2000). If AM fungi do not get close to the root of a host plant in this limited time, growth will stop accompanied by germ-tube septation and nuclear autolysis (Bago et al., 1998). The arrested hyphal growth could restart several times which is believed to be a strategy to increase the chance to find a host (Koske, 1981; Bago et al., 2000).

Other important propagules are pre-infected root fragments. Hyphal grow out from root fragments is frequently observed and probably based on the presence of vesicles that store large amount of lipids (Powell, 1976; Biermann and Linderman, 1983; Brundrett et al., 1999; Klironomos and Hart, 2002). It is, however, worth noting that the AM fungus *Scutellopora calospora*, which does not form vesicles in roots, also has the ability to grow out from pre-infected root fragments (Tommerup and Abbott, 1981). The vitality of root fragments differs and is influenced by factors e. g. time, soil animals and soil disturbance (Thompson, 1987; Friese and Allen, 1993; Merryweather and Fitter; 1998).

Hyphal networks from infected plants are able to establish rapid and steady mycorrhization of roots developing from newly germinated seedlings (Olsson et al., 2002; Leake et al., 2004;Simard and Durall, 2004). With the support of host plants, hyphae grow fast and keep the ability of infection (Camel et al., 1991; Powell, 1979). Pre-established hyphal networks are much more efficient in nutrient exploration and delivery than newly developed mycelia. The extension of such networks is influenced by factors such as soil texture, soil microorganisms and agricultural practices (McGonigle and Fitter, 1988; Pattinson et al., 1997; Drew et al., 2003, 2005). Using a 'nursery plant'-supported AM fungus, the infection of a

target plant can reach maximal colonization after ten days (Rosewarne et al., 1997), and this facilitates infection studies e. g. of mycorrhiza mutant plants (Feddermann et al., 2010; Javot et al., 2011).

1.2.2 Accommodation of AM fungi and recruitment of the common symbiosis mechanism by root nodule symbiosis

1.2.2.1 Different types of nodule symbiosis

In natural environment, plants can form different endosymbioses with microorganisms (Parniske, 2000). Besides arbuscular mycorrhiza, root nodule is the most studied and best understood plant symbiosis (Udvardi and Day, 1997). Root nodule symbiosis (RNS) can effectively supply host plants with nitrogen. Atmospheric N_2 is reduced to ammonia and exchanged against reduced carbon compounds (mainly dicarboxylates) from host plants to support bacterial metabolism (Udvardi and Day, 1997). In contrast to AM, the nodule symbiosis is restricted to four related orders within the clade Eurosideae of the angiosperms (Soltis et al., 1995). There are two main types of RNS formed between different bacteria with different host plants. Fabaceae (except lupin) interact with a phylogenetic diverse group of Gram-negative bacteria called rhizobia (Kistner and Parniske, 2002; Bapaume and Reinhardt, 2012), while plants among the Fagales, Cucurbitales and Rosales form actinorhiza with nitrogen-fixing actinobacteria of the genus *Frankia* (Markmann and Parniske, 2009). A distinctive feature of the legume-rhizobia symbiosis is its high level specificity which appears at the early stage during bacterial infection and nodule formation, as well as at the late stage of nitrogen fixation (Wang et al., 2012). Model nodule symbioses are the interactions between *Medicago truncatula* and *Sinorhizobium meliloti*, and between *Lotus japonicus* and *Mesorhizobium loti*.

1.2.2.2 The common *SYM* genes in early stages of arbuscular mycorrhiza and root nodule symbiosis

Based on molecular clock data, diversification of AM fungi occurred about 460 million years ago and, the earliest AM-like structures were found in about 400 million years old fossils. Nodule-like structures were, however, dated 65 million years ago (Kistner and Parniske, 2002) (**Figure 1.1**). Although function and morphology are different between these two symbioses, molecular and genetic data suggested that the formation of nodule symbiosis recruited

mechanisms evolved earlier in the AM symbiosis (Kistner and Parniske, 2002; Kistner et al., 2005).

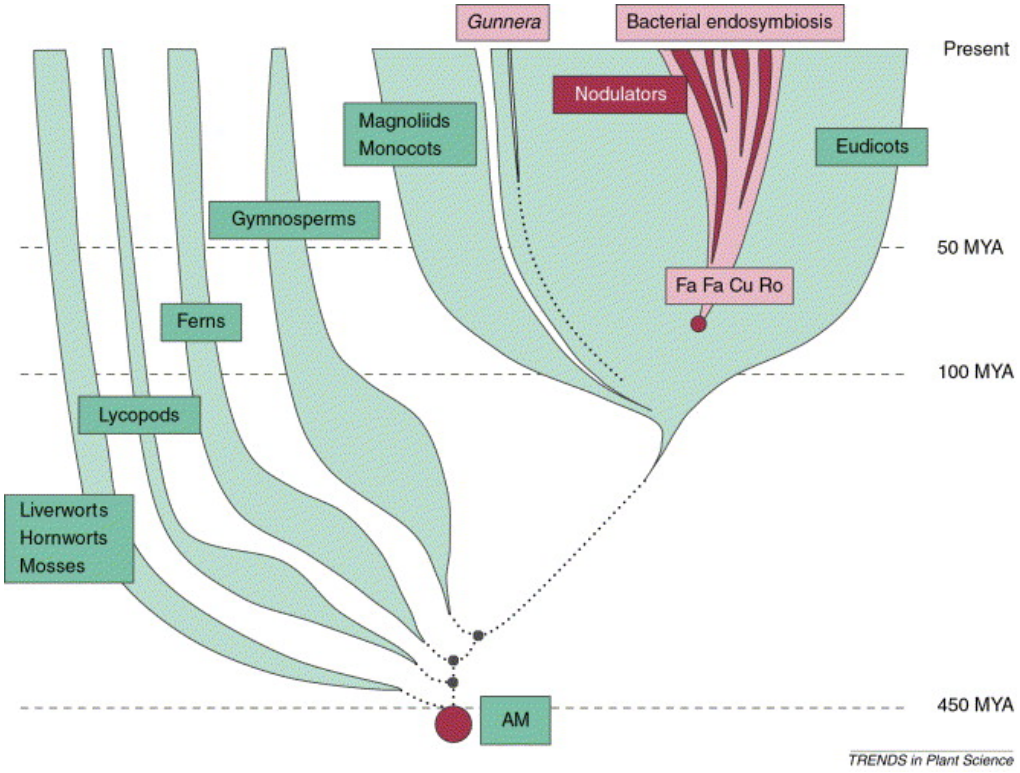

Figure 1.1 Evolution of plant root endosymbiosis (adopted from Kistner and Parniske, 2002).

Before physical contact, diffusible signal molecules (strigolactones and flavonoids) that are secreted by plant roots can be perceived by AM fungi (Aikyama et al., 2005) and rhizobia (Firmin et al., 1986). In return, rhizobia produce specific symbiotic signals called NOD factors (NFs) that are lipochitooligosaccharides (LCOs) (Hassan and Mathesius, 2012), and AM fungi secret similar LCOs signal molecules called MYC factors (MFs) (Maillet et al., 2011). LCOs have a backbone of β-1, 4-linked *N*-acetylglucosamine, a structure shared with chitin and peptidoglycan, the major components of fungal and bacterial cell walls (Bapaume and Reinhardt, 2012). Perception of NFs and MFs requires receptors that are located on the plasma membrane and consist of an extracellular domain with lysin motif (LysM) repeats functional for signal binding and an intracellular protein kinase domain responsible for activating a particular signal transduction chain (Arrighi et al., 2006; Lohmann et al., 2010). A homologue of the NOD factor receptor (NFR), which recognizes NFs in legumes, was identified in the non-legume *Parasponia andersonii* (Cannabaceae). This homologue (PaNFR) possesses a dual function as receptor for both NFs and MFs (Op Den Camp et al., 2011). The

presence of the recently gained primitive symbiosis of *Parasponia* with rhizobia, phylogenetically distinct from the legume nodules, indicates that the NF perception was recruited from the evolutionary older AM recognition mechanism (Op Den Camp et al., 2011). This mechanism later diversified and resulted in separated receptors for MFs and NFs in legumes. Moreover, plants have receptors for chitin and peptidoglycan to induce downstream defense reaction against pathogenic fungi and bacteria, and these receptors might also be derived from the ancestors being functional in the AM symbiosis. Such receptors might have lost their affinity for LCOs, because AM fungi and rhizobia do not induce strong defense responses. Alternatively upon recognition of AM fungi or rhizobia, plants suppress their defense responses and actively allow symbiotic interactions (Bapaume and Reinhardt, 2012).

Perception of NFs or MFs activates a signal transduction chain which finally leads to accommodation of AM fungi or rhizobia. The genes that encode the components of this signal transduction chain for both AM and RNS, are designated as the common *SYM* genes(Kistner and Parniske, 2002). At least seven genes were up to now identified in *L. japonicus* and four identified in *M. truncatula* (*DMI1*, *DMI2*, *DMI3,* and *IPD3*) (Parniske, 2008; Horváth et al., 2011).

SYMRK, which is known as *DMI2* in *M. truncatula* and *NORK* in *M. sativa* (Endre et al., 2002; Stracke et al., 2002), encodes a symbiosis receptor kinase which is the first component of signal transduction (Parniske, 2008). At least three different versions of SYMRKs exist in the angiosperm lineages which differ in length and domain structure. Long version SYMRKs that exist in nodule-forming plant species are composed of a long N-terminal extracellular region (NEC domain) and three leucine-rich repeat (LRR) motifs (Markmann et al., 2008). Shorter SYMRK versions that lost one LRR motif or NEC domain and a LRR domain are only competent for functioning in AM formation (Markmann et al., 2008). The fact that long version SYMRKs from non-nodule plant species can restore interactions with AM fungi and rhizobia in a *SYMRK* mutant, while the short version only restores AM formation, and the function of *SYMRK* in the Actinorhizal symbiosis confirms the central role of *SYMRK* in signal transaction and is another indication for the recruitment of RNS from AM (Markmann et al., 2008).

After perception of NFs or MFs, calcium spiking triggers host cell transcriptional reprogramming in both mycorrhization and nodulation (Oldroyd and Downiw, 2006). Calcium is a common secondary messenger in diverse signaling pathways. Ca^{2+} concentration

oscillation in the cytoplasm and nucleoplasm around the nuclear envelope is termed calcium spiking, which consists of two phases: a rapid increase of calcium levels, and a more gradual decline (Oldroyd and Downiw, 2006). *CASTOR* and *POLLUX* (known also as *DMI1* in *M. truncatula*) are needed to generate the nucleus-associated calcium spiking (Peiter et al., 2007; Imaizumi-Anraku et al., 2005), and their mutation results in the failure of calcium spiking and in symbiosis abortion. The proteins CASTOR and POLLUX share similar domain structure and high sequence similarity (Imaizumi-Anraku et al., 2005). The encoded proteins are located in the nuclear membrane and function as potassium channels regulating the export of calcium from the perinuclear space into the surrounding (Peiter et al., 2007). In addition to *CASTOR* and *POLLUX* or *DMI1*, a number of nucleoporins also play an important role upstream of calcium spiking. Three genes that belong to the common *SYM* genes were identified in *L. japonicas* (nucleoporin85, nucleoporin133 and NENA), and their mutation leads to defective calcium spiking and aborted symbiosis (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010). Their precise role is, however, unknown.

A calcium-calmodulin-dependent protein kinase (CCaMK) decodes the calcium spiking that is induced during AM or RNS formation (Oldroyd and Downiw, 2006). The calmodulin-binding domain and calcium-binding EF hand motif of CCaMK (known also as DMI3 in *M. truncatula*) give the calcium sense ability to this protein (Kosuta et al., 2008; Levy et al., 2004). The substrate protein of CCaMK is encoded by *CYCLOPS* in *L. japonicus* and *IPD3* in *M. truncatula* (Yano et al., 2008; Messinese et al., 2007; Horváth et al., 2011). The *CYCLOPS* encoded protein contains a functional nuclear localization signal and a carboxy-terminal coiled-coil domain (Parniske, 2008). Phosphorylation of this protein is required for the downstream transcriptional reprogramming in the symbiosis (Horvath et al., 2011; Yano et al., 2008; Messinese et al., 2007). Mutation of *CYCLOPS* severely impairs both symbioses processes and particularly arbuscule formation (Kistner et al., 2005).

1.2.2.3 The similar root behave in AM fungi and rhizobium guidance

After the first steps of communication between roots and the two types of microorganisms and further signaling events, particular responses of root cells guide AM fungi or rhizobia to their target cells.

AM fungal hyphae, after recognition of the plant signal, do not grow towards the plant (no chemotaxis), but instead start to ramify extensively (presymbiotic development) which increases their probability to contact the surface of plant roots (Tamasloukht et al., 2003).

Upon physical contact, instead of direct penetration, AM hyphae grow along root surface and form hyphopodia (Bastmeyer et al., 2002; Genre et al., 2005; Parniske, 2008). Before AM fungal hyphae enter root epidermis cells, a transcellular structure designated the pre-penetration apparatus (PPA) is formed right underneath the hyphopodia (Genre et al., 2005). This PPA is a cytoplasmic column containing microtubule and microfilament bundles, dense endoplasmic reticulum cisternae and a central membrane thread. Only when the PPA is formed, fungal hyphae are able to enter epidermal cells (Genre et al., 2005). This PPA being similar to the pre-infection thread in RNS (see below) guides AM fungal hyphae across the outer cortex to the inner cortical cells, where it is also involved in the intracellular development of fungal arbuscules (Genre et al., 2005, 2008). Mutation of the common *SYM* genes (*DMI2* and *DMI3* in *M. truncatula*) inhibits PPA development (Genre et al., 2005, 2008; Siciliano et al., 2007).

Rhizobia, after recognition of a legume plant, are physically attached to plant root hairs and enclosed by either individual root hair tip curling or by contact between adjacent root hairs forming an apoplastic space (Fournier et al., 2008). A host-derived inwardly growing tubular hollow structure called infection thread (IT) guides the rhizobia. Followed behind the downward-migrating root hair nucleus, the IT grows continuously ahead of the proliferating rhizobia. The infection thread traverses the entire root hair and cortical cells and reaches the inner cortex (Fournier et al., 2008). In cortical cell layers, transcellular cytoplasmic bridges (also called pre-infection threads) define the pathway of the progressing IT (Van Brussel et al., 1992).

1.2.2.4The similarity of plant-derived membrane in AM and RNS accommodation

The symbiosome is the main characteristic compartment of root endosymbioses. In this compartment within the host cell a particular plant-derived membrane partially or completely encloses the symbiotic microorganisms (Parniske, 2000). This plant-derived periarbuscular or peribacteroid membrane is specialized for diverse functions in molecular communication and nutrient exchange (Bapaume and Reinhardt, 2012).

Guided by the PPA, AM fungal hyphae penetrate the root epidermis and reach inner cortical cells where PPA-like bridges link the enlarged plant nucleus with the fungal hyphae until the arbuscule trunk is formed (Genre et al., 2008). ER-rich cytoplasmic aggregations then organize at distinct sites where the lateral hyphae will branch until the differentiation of the

fungal arbuscule is finished (Genre et al., 2008). Although the plant-derived membrane is continuous with the cortical cell plasma membrane, the periarbuscular membrane is composed in a different way forming two distinct domains (arbuscule branch domain and arbuscule trunk domain) based on the protein composition (Pumplin and Harrison, 2009). Functional nutrient transporters, for instance the mycorrhiza-specific phosphate transporter MtPT4 in *M. truncatula*, are specifically located on the arbuscule branch domain (Javot et al., 2007b; Pumplin and Harrison, 2009).

In the developing nodule symbiosis, the PIT guides rhizobia inside the infection thread from which they are finally released inside the nodule primordium. Here the bacteria enter the cortical cells by an endocytosis-like process and the symbiosome is formed with the plant-derived peribacteroid membrane (Parniske, 2000). Rhizobia inside cortical cells then divide and differentiate to form the nitrogen-fixing bacteroids (Udvardi and Day, 1997).

Similar to the membrane in arbusculated cells, the peribacteroidmembrane facilitates the exchange of fixed nitrogen and carbohydrates, and is also the site of communication between rhizobia and host plants. It was therefore hypothesized that the regulatory pathway forming the periarbuscular membrane was recruited by RNS. Direct evidence came from the finding of two homologous exocytotic Vesicle-Associated Membrane Proteins in *M. truncatula* (MtVAMP72d and MtVAMP72e) that are in charge of membrane formation for both AM and legume-rhizobium symbiosis (Ivanov et al., 2012). In addition, Vapyrin, a VAMP-associated protein and its homologs were found in almost all plant species including *A. thaliana*, affects both AM fungi and rhizobia symbiosis (Pumplin et al., 2010; Murray et al., 2011). Additional indirect evidence is the fact that the membrane surrounding the fixation thread in *Parasponia* is connected with the plasma membrane like the periarbuscular membrane (Webster et al., 1995).

1.2.3 Extension of intra- and extra-radical hyphae

Reports on arbuscular mycorrhizas go back to the nineteenth century and different types were separated based on morphological variations (Frank, 1885, 1887; Janse, 1897; Petri, 1903). At the start of twentieth century, two types of arbuscular mycorrhizas were defined: the *Arum*- and the *Paris*-type named after their first observation in the roots of *Arum maculatum* and *Paris quadrifolia* (Gallaud, 1905). For the *Arum*-type, fungal hyphae grow in the intercellular space between root cortical cells, and after a single penetration of the cell wall usually one

highly branched intracellular structures called arbuscule is formed (the Latin word "*arbusculum*" means bush or little tree). For the *Paris*-type, the intercellular phase of colonization is highly restricted or completely absent. The hyphae grow directly from cell to cell, and form extensive intracellular hyphal coils which can carry several little arbuscules per cell (Smith and Smith, 1997). At the early beginning, only *Arum*-type were identified as AM while *Paris*-type have been ignored due to the lack of the previously described "typical" AM structures (Smith and Read, 2008). Based on a thorough literature survey, Smith and Smith (1997) reported that *Paris*-type occurs more often in the plant kingdom than *Arum*-type. Interestingly, most cultivated herb plants form *Arum*-type mycorrhizas while in many trees and forest herbs *Paris*-type mycorrhizas are detected. In a recent study, in which twelve different plants were inoculated with six AM fungi from different genera, arbuscule morphology ranged as a continuum from *Arum*-type to *Paris*-type but mycorrhizal structures did not vary during the course of colonization (Dickson, 2004). The mycorrhizal type is believed to be determined by both plant and its AM fungal partners while the environmental conditions do not exert any influence (Dickson, 2004). For instance, in tomato roots, both *Arum*-type and *Paris*-type mycorrhizas were found with different AM fungal species (Cavagnaro et al., 2001) while *Rhizophagus irregularis* (former called *Glomus intraradices*) forms both, an *Arum*-type mycorrhiza in tomato and a *Paris*-type mycorrhiza in *Panax quinquefolius* roots (Cavagnaro et al., 2001; Armstrong and Peterson, 2002), and *Gigaspora rosea* develops even both types in the same rice root (Kobae and Hata, 2010).

In *Arum*-type mycorrhiza, AM fungal hyphae penetrate root cell walls and first form the arbuscular trunks, which never penetrate, but invaginate cell plasma membranes (Cox and Sanders, 1974; Bonfante-Fasolo, 1984). With the guidance of PPA-like structures, thin hyphae growing from the trunk branches and form the fully developed arbuscule (Genre et al., 2008). As the arbuscule itself, the periarbuscular membrane can be separated into two different domains with different functions (Pumplin and Harrison, 2009) and those parts surrounding the fine branches are suggested to be the site of mineral nutrient uptake into the plant cell (Gianinazzi-Pearson et al., 1991; Harrison et al., 2002; Javot et al., 2007b; Kobae and Hata, 2010). Together with membrane development, a layer of material with the same composition as the primary wall of plant cell is deposited around the trunk of the arbuscules, gradually becoming thinner with arbuscule development and disappeared before reaching the fine branches (Smith and Read, 2008). Arbuscules that are relatively short-living structures last about seven days in fast growing species while appear longer lived in slow growing

woodland plants (Scannerini and Bonfante-Fasolo, 1983; Bonfante-Fasolo et al., 1984; Alexander et al., 1988; Brundrett and Kendrick, 1990a; Peterson and Bonfante, 1994). Phosphate transportation across the plant-fungal interface in the arbusculated cells of rice roots last only about two days indicating a relatively short existence of functional arbuscules (Kobae and Hata, 2010). With the collapse of the branches arbuscule degeneration starts and this process is faster than formation. Finally the senescing arbuscule is separated by a septum formed between the arbuscule trunk and the intercellular hypha (Javot et al., 2007a; Kobae and Hata, 2010). In *Paris*-type mycorrhiza, hyphae develop intracellularly with the same speed of hyphal development inside host roots as *Arum*-type mycorrhiza (Dickson, 2004). In confocal microscopy observation, the coils of *Paris*-types mycorrhiza have a greater surface area than the arbuscules formed by *Arum*-type mycorrhiza (Dickson and Kolesik, 1999). As the coils were formed in consecutive cells, the fungal biomass of *Paris*-type mycorrhization per infected root length was much higher than the *Arum*-type in which the arbuscules were much more scattered (Cavagnaro et al., 2003). Hyphal coils develop prior to arbusculated branches (Cavagnaro et al., 2001), but coils and arbusculated coils are believed to play a similar function in phosphate transportation (Karandashov et al., 2004). In addition to arbuscules, different shaped thick-walled vesicles may be formed in mycorrhizal roots which gave the symbiosis the former name vesicular-arbuscular mycorrhiza. They may be storage organs, may also serve as propagules and their formation is affected by environmental conditions (Smith and Read, 2008). Such vesicles are, however, not formed by species from the Gigasporaceae (*Scutellospora* and *Gigaspora*). The general extent of intraradiceal hyphae spread is influenced by genetic factors such as the fungal isolate and the plant species or even cultivar and by the environment (Smith and Read, 2008).

Intraradical hyphae probably do not grow out of plant roots. It is generally agreed that the extraradical hyphae start from existing hyphae outside the root by extension and branching, but their growth depends on the establishment of the intraradical mycelium (Bécard and Piché, 1989; Kling et al., 1996; Poulsen et al., 2005). Other study confirmed this by demonstrating the transportation of triacylglycerol, which is the main form of carbon in AM fungi from intraradical mycelium to extraradical mycelium (Bago et al., 2002). One important function of the extraradical hyphae is mineral nutrient uptake such as phosphate and nitrogen (Harrison and van Buuren, 1995; Maldonado-Mendoza et al., 2001; Benedetto et al., 2005; Govindarajulu et al., 2005). Another function of extraradical hyphae is to form new infection sites with the same or neighboring plants, by which mineral nutrients as well as signals can be

exchanged (Meding and Zasoski, 2008; Song et al., 2010). One important function of the extraradical hyphae can also be the secretion of the glycoprotein Glomalin. This protein affects soil aggregation changing physical characters of the mycorrhizosphere (Wright and Upadhyaya, 1998). After a certain time of growth, extraradical hyphae will produce spores that contain large amounts of lipids and function as the start of the next generation. Different factors such as growth time, fungal species, and environmental conditions influence sporulation (Smith and Read, 2008).

1.3 Mineral nutrient uptake in the AM symbiosis

The main character of the interaction between AM fungi and host plants is the exchange of nutrients. As obligate biotrophs, AM fungi receive photosynthates from their hosts while supplying them with inorganic nutrients. Extracellular hyphae can exceed 100 meters per cubic centimeter soil, and can spread out of the nutrient depletion zone into the surrounding of the rhizosphere (Smith et al., 2011).

1.3.1 Phosphorus and AM

Phosphorus is a major macronutrient for all organisms. It serves multiple functions as a key structural element in nucleic acids, phospholipids and several enzymes and coenzymes. Its importance is also revealed in energy metabolism, activation of metabolic intermediates, signal transduction cascades and enzyme regulation (Karandashov and Bucher, 2005). The direct uptake pathway of plants is by the absorption via the root epidermis and the root hairs. Although large amounts of phosphorus exist in soils, most of it is in a low soluble form as phosphates of iron, aluminum, and calcium. When the phosphates absorption rate of the direct pathway exceeds the diffusion rate of soluble phosphate, a depletion zone will form around the root, and this limits further phosphate uptake (Smith and Read 1997).

Several strategies are developed by plants to cope with phosphorus deficiency. A common response is to increase the root/shoot ratio, using the expense of shoot growth to stimulate root growth (Hermans et al., 2006). Another strategy is to change the growth of different root types (Hodge 2009). For instance, when *Arabidopsis thaliana* grows under phosphorus stress, the number and length of lateral roots will be induced and primary root growth will be suppressed (Pérez-Torres et al., 2008). In plants, two phosphate uptake systems have been identified (high-affinity system and low-affinity system). When plants grow under phosphorus deficiency conditions, the high-affinity phosphate transport system acquiring

phosphate against concentration gradients is activated (Rausch and Bucher, 2002), as phosphate concentration in plant root cells can be up to 1000-fold higher than in the surrounding soil solution. Alternative solutions include at first, solubilization of phosphate trapped in complexes by secreting organic acids like malate and citrate to compete for cation-binding partners, secondly, mineralization of phosphate from organic compounds by secreting phosphatases, and thirdly, taking advantage of bacteria that solubilize phosphate in the rhizosphere.

polymer of three to thousand phosphates connected by high-energy phosphoanhydrate bonds and serves multiple functions from phosphate storage to improve translation fidelity (Kornberg et al., 1999; McInerney et al., 2006). Following the formation, poly-P is then translocated in the vacuolar compartment from the extraradical to the intraradical hyphae (Javot et al., 2007a; Hijikata et al., 2010). Particular enzymatic activities or expressed genes involved in the process of the formation of poly-P and the translocation inside the vacuole were identified in the hyphae (Tatsuhiro et al., 2001; Tani et al., 2009; Tisserant et al., 2012). An evidence for massive poly-P translocation is the direct correlation between root poly-P content and root colonization level (Ohtomo et al., 2005). The shorter length of the poly-P chain in the intraradical hyphae than in the extraradical hyphae suggests that poly-P is hydrolyzed in order to release phosphate to the plant (Ohtomo et al., 2005). The fungal alkaline phosphatase (ALP) had been considered to be involved in the phosphate efflux from intraradical hyphae and, its activity was a useful marker for analyzing symbiotic efficiency (Tisserant et al., 1993; Kojima and Saito, 2004; Javot et al., 2007a). Once the phosphate is released in the intraradical hyphae, it is assumed to be transferred to the periarbuscular apoplastic compartment. So far the mechanism behind this process is unknown. As the concentration gradient is probably the basis for the phosphate passage across the fungal plasma membrane, the transfer could be facilitated by ion-specific carriers, pumps or channels (Karandashov and Bucher, 2005). The phosphate inside the periarbuscular apoplastic compartment is available for the plant, but the amount of uptake might be also controlled by the mycorrhizal fungi, as arbuscules expressing *GmosPT* or *GintPT* could principally re-absorb phosphate from this compartment (Benedetto et al., 2005; Balestrini et al., 2007). This could contribute to the ‘reciprocal benefit in the mycorrhizal symbiosis’ (Kiers et al., 2011) stabilized by the control of nutrient exchange by both partners. The last step of the mycorrhizal pathway is the phosphate transport from the periarbuscular space into the cortex cell by plant transporters. Expression studies revealedthat particular phosphate transporter genes from different species e. g. tomato, potato, rice, barley, medic, maize, tobacco, and petunia are specifically induced in mycorrhizal roots (Javot et al., 2007b; Bucher, 2007; Breuillin et al., 2010; Loth-Pereda et al., 2011; Tan et al., 2012). Promoter::GUS fusion and *in situ* hybridization indicated moreover that at least the mycorrhiza-specific transporters in tomato, potato, medic, maize, barley and poplar were dominantly or even exclusively expressed inside the cells harboring fungal arbuscules (Javot et al., 2007a; Loth-Pereda et al., 2011). Observed via live-cell imaging, the mycorrhizal specifically induced phosphate transporter in *M. truncatula* was localized on the ‘arbuscule branch domain’ of the

periarbuscular membrane (Pumplin and Harrison, 2009). Silencing of the genes encoding these phosphate transporters resulted in much less branched arbuscules and reduced phosphate uptake in different plants including *M. truncatula*, *L. japonicus* and *Solanum lycopersicum* (Nagy et al., 2005; Maeda et al., 2006; Javot et al., 2007b).

In mycorrhizal plants, direct and mycorrhizal pathways are functioning at the same time, but in different root areas and contribute to phosphate nutrition to different extents. When plant roots with their apex including epidermis and root hairs grow into non-depleted soils, the direct pathway will be more effective. After root hairs disappeared and the depletion zone is formed, the mycorrhizal pathway will take over phosphate uptake (Smith et al., 2011). Under low phosphate conditions, the mycorrhizal pathway is thought to dominate the phosphate uptake, but the contribution of different mycorrhizal fungi varies. Usually phosphate uptake via the mycorrhizal pathway is mirrored by improved plant growth of mycorrhizal versus control plants. By application of labeled phosphate, Smith et al. (2004), however, demonstrated that even in growth-depressed plants, the mycorrhizal fungus supplied phosphate. With increased phosphate supplements in fertilizers, the contribution of the mycorrhizal pathway declined while the direct uptake increased (Nagy et al., 2009). Although under high phosphate conditions, colonization decreases, AM fungi still provide a large proportion of phosphate to wheat, barley and tomato while overall phosphate uptake did not change. The hidden phosphate uptake via the mycorrhizal pathway indicates declined function of the direct pathway compared with non-mycorrhizal plants (Smith et al., 2004). The declined function of the direct way is thought to be the result of down-regulation of transporter genes and the formation of a depletion zone around the actively absorbing root (Smith et al., 2003).

1.3.2 Coupled phosphate and carbohydrate exchange in AM

While supplying phosphate, AM fungi receive carbohydrates from host plants. According to Smith and Read (2008), AM fungi can obtain up to 20% of the photoassimilates. Sucrose as a stable disaccharide is the main form how carbohydrates are transported during the flow through the plant via phloem vessels (Hohnjec et al., 2003). In an axenic compartment experiment, application of labeled sucrose to the mycorrhizal root stimulated carbohydrate flux towards the fungus as the biomass of extraradical hyphae was significantly increased and, these hyphae contained large amounts of labeled carbon (Bücking and Shachar-Hill, 2005). Sucrose as carbon source cannot be directly utilized by AM fungi and needs to be hydrolyzed

to hexoses which then can be taken up by AM fungi (Schubert et al., 2004). A model for the interaction between carbohydrates and phosphate fluxes in the AM symbiosis was proposed by Bücking and Shachar-Hill (2005) (**Figure 1.2**), in which the exchange of carbohydrates and phosphate is coupled. A number of genes and encoded enzymes and transporters that are involved in the exchange of carbohydrates and phosphate have been discovered. On the plant side, genes encoding sucrose transporters (*SISUT1*, *SISUT2*, and *SISUT4*) involved in sucrose transport across membranes were shown in tomato plants to be mycorrhizal up-regulated in, both leaves and roots (Boldt et al., 2011). *MtSucS1* encoding a sucrose synthase that is responsible for the synthase of sucrose and generating sink strength was found to be highly activated in root regions containing arbuscules (Hohnjec et al., 2003). The activity of cell wall invertase that is in charge of cleaving sucrose and the corresponding gene expression are increased in mycorrhizal roots especially in colonized cells (Schaarschmidt et al., 2006; García-Rodríguez et al., 2007; Tejeda-Sartorius et al., 2008). In arbuscule-harboring cells, the expression of plant genes for a phosphate transporter (*MtPT4*) and for an electrochemical gradient-generating H^+-ATPase (*MtHA1*) was detected (Harrison et al., 2002; Krajinski et al., 2002). Similarly, two genes encoding ATPases in charge of the electrochemical gradient enabling nutrient transfer across the plasma membrane and the monosaccharide transporter gene (*MST2*) were identified on the fungal side (Requena et al., 2003; Helber et al., 2012). According to the carbohydrate – phosphate exchange model, when AM fungi provide the plant with more phosphate, the plant rewards the AM fungus with more carbohydrates and vice versa (Kiers et al., 2011). This predicts that if the exchange of carbohydrates and phosphates is disturbed, the formation and function of arbuscular mycorrhiza will be affected. Indeed in tobacco plants, overexpression of an invertase inhibitor gene led to decreased fungal development inside the root (Schaarschmidt et al., 2007) and, knockdown of *MtSucS1* in *M. truncatula* resulted in the down-regulation of AM marker genes and a reduced number of arbuscules with an increasing proportion of degenerated and dead arbuscules (Baier et al., 2010). Overexpression of one sucrose transporter gene (*SoSUT1*) involved in phloem loading of photoassimilates increased the colonization of an AM fungus even under high phosphate condition while the reduced expression of *SoSUT1* also reduced the contribution of phosphate uptake via mycorrhiza (Gabriel-Neumann et al., 2011). When *MtPT4* in *M. truncatula* was silenced or mutated, arbuscules died inside root cells before maturation and the fungus could not develop (Javot et al., 2007b, 2011). In roots with silenced fungal *MST2* , arbuscules were either not fully developed or showed premature senescence and the function of phosphate

transport was almost completely abolished (Helber et al., 2011). All these evidences support the idea that nutrient exchange is the basis of a successful AM symbiosis.

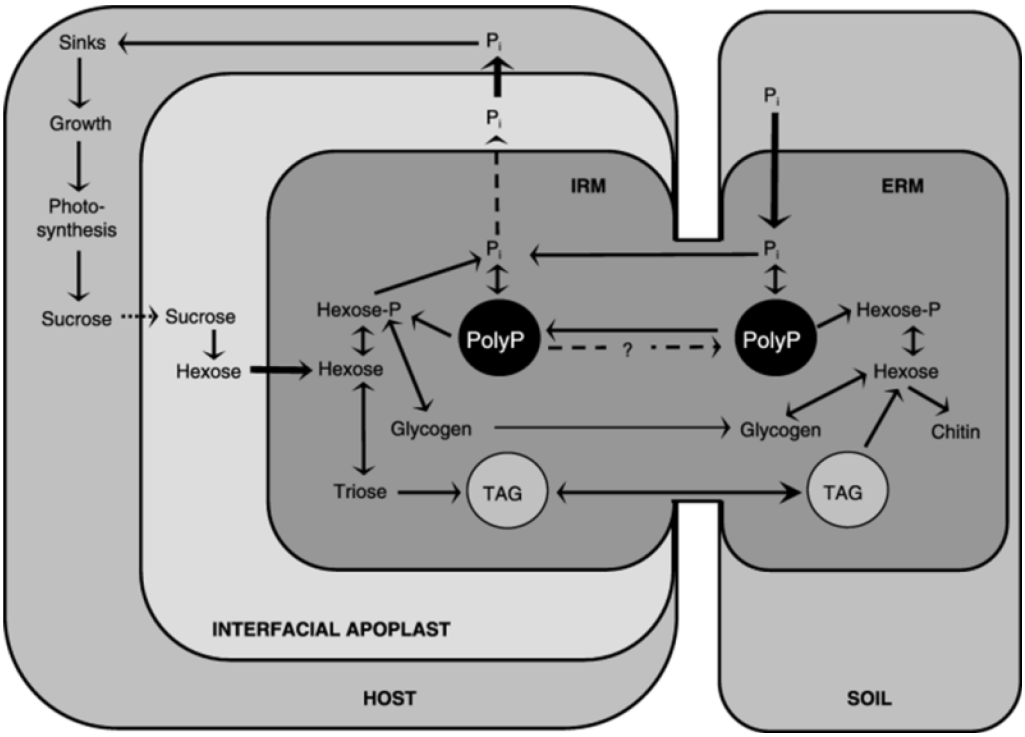

Figure 1.2 Coupled phosphate (P_i) and carbohydrate exchange in the arbuscular mycorrhizal (AM) symbiosis (adopted from Bücking and Shachar-Hill, 2005). Bold arrows, active uptake processes; broken arrows, passive efflux processes. ERM, extraradical mycelium; IRM, intraradical mycelium; polyp, polyphosphates; TAG, triacylglycerol.

1.3.3 Nitrogen uptake and AM

Nitrogen is the most important mineral nutrient in plants mainly due to its role in the synthesis of proteins (Marschner, 1995). Organic nitrogen accounts for most part of nitrogen in soil, and its mineralization needs microorganism. Released ammonium and its nitrified form nitrate are the two inorganic nitrogen forms that plants can utilize (Miller and Cramer, 2004). The availability of nitrogen usually limits plant growth and, AM fungi are believed to contribute to nitrogen uptake due to their fast and massive extraradical hyphae exploration of the soil (Ames et al., 1983; George et al., 1995; Hawkins and George, 1999; Hawkins et al., 2000). Hyphae of AM fungi have the capability of absorbing both ammonium and nitrate. Genes that encode ammonium and nitrate transporters were identified and their expression was shown to be induced in extraradical hyphae under low nitrogen concentrations (López-Pedrosa et al., 2006; Tian et al., 2010; Pérez-Tienda et al., 2011). Nitrate has to be reduced and genes encoding nitrate reductase could be detected in AM fungi (Kaldorf et al., 1994, 1998). After

the uptake of ammonium or nitrate and the reduction of nitrate, nitrogen is assimilated via glutamine synthase and glutamine oxoglutarate aminotransferase(GS-GOGAT cycle), resulting in arginine (Govindarajulu et al., 2005). Arginine is then transported from the extraradical into the intraradical hyphae where it is broken down. The resulting ammonium is released into the periarbuscular space and taken up by host plant ammonium transporters (Jin et al., 2005). A model of nitrogen uptake, transport and transfer by AM fungi was published by Jin et al. (2005), which also indicates how carbon could be recycled (**Figure 1.3**). Besides from soil-originated nitrogen, the fixation of atmospheric N_2 in nodules is another important nitrogen source (see above). The enhancement of nodule functioning by arbuscular mycorrhiza due to improved phosphate and micronutrient supply (Kawai et al., 1986; Bethlenfalvay, 1992) is believed to be an indirect effect of the mycorrhizal symbiosis on nitrogen nutrition of plants (Smith and Read, 2008).

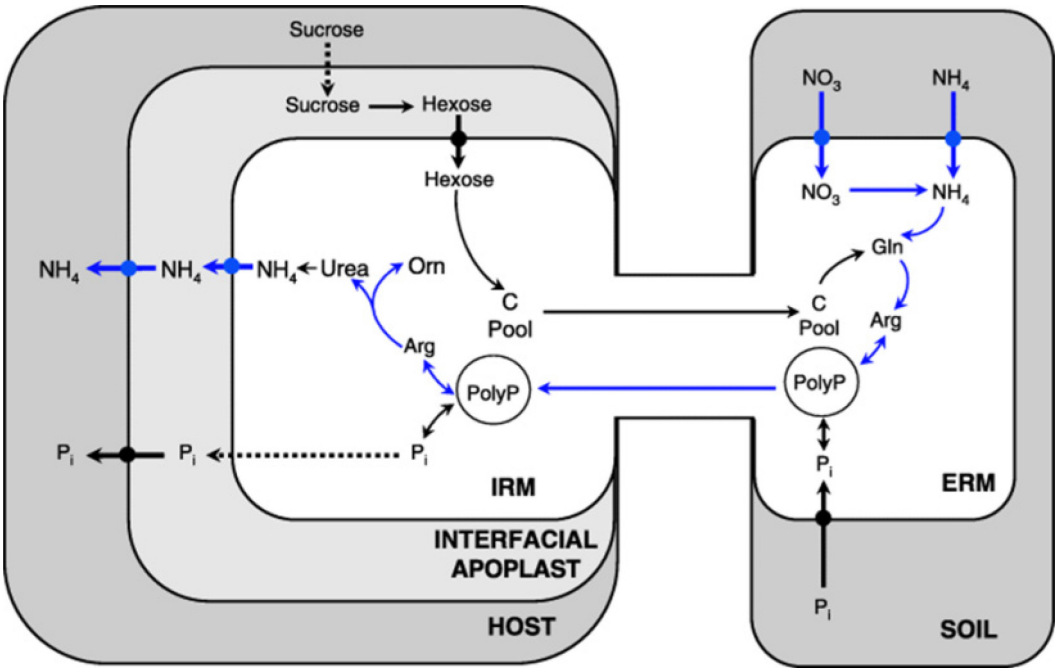

Figure 1.3 The model of N transport and metabolism in the symbiotic system between transformed carrot root and arbuscular mycorrhizal fungi (adapted from Jin et al., 2005). ERM, extraradical mycelium; IRM, intraradical mycelium; Arg, arginine; Pi, orthophosphate; PolyP, polyphosphate.

1.3.4 Other nutrient uptake and AM

Besides phosphate and nitrogen, the uptake of other plant mineral nutrients such as sulfur, potassium, copper and zinc can also be improved by AM fungi (Marschner and Dell, 1994). As an essential nutrient in plant, sulfur is involved in the biosynthesis of different molecules

such as amino acids, membrane sulpholipids, cell walls and secondary metabolites in plants (Saito, 2004; Foyer and Noctor, 2009; Popper et al., 2011). In a ^{35}S-labelling experiment, in which carrot roots and an AM fungus were grown monoxenically, the mechanism behind the sulfur uptake and transfer from the fungus to the host plant was studied (Allen and Shachar-Hill, 2009). The sulfate uptake via the AM fungus was inversely related to root uptake and repressed by the presence of sulfur-containing proteins (Allen and Shachar-Hill, 2009). Identification of sulfate transporter genes that show differential RNA accumulation upon mycorrhization implied the existence of a direct and a mycorrhizal sulfur uptake pathway (Casieri et al., 2012). Although AM fungi can supply plants with sulfur, the reliance of AM symbiosis on sulfur is not as strong as phosphate and nitrogen based on the finding of an unmodified arbuscular phenotype in *MtPT4* silenced plant under sulfur-depriving condition (Javot et al., 2011). Moreover, AM-reduced sulfur stress responses were only observed when the phosphate demand of the host plant was fulfilled (Sieh et al., 2013).

The improvement of potassium in plant shoots but not roots under phosphate deficient conditions while the diminished uptake under phosphate sufficient conditions implies an indirect effect of AM on potassium uptake (Smith et al., 1981; Rhodes and Gerdemann, 1978). The depletion of potassium in hyphal compartments indicates the possibility of direct uptake improvement by AM fungi (George et al., 1992). For the uptake of copper, the effect of AM can be up to 62% of total uptake but in contrast to potassium the AM effect did not depend on phosphate nutrition (Li et al., 1991). Although copper uptake in AM plants was confirmed, the mechanism is so far uncertain (Smith and Read, 2008). Another studied nutrient was zinc which is essential for plant growth and is deficient in about 30% of soils world-wide (Brown and Wuehler, 2000; Kochian, 2000). AM fungal inoculation alleviates Zn deficiency in different plants (Lu and Miller, 1989; Faber et al., 1990; Kothari et al., 1991; Wellings et al., 1991; Bürkert and Robson, 1994), and the uptake via AM fungal hyphae was confirmed in a study using isotope-labeled zinc (Cooper and Tinker, 1978). Little knowledge on the mechanism of improved zinc uptake so far exists. A zinc transporter gene identified in an AM fungus seems to play a role of protecting fungus against too high zinc concentrations rather than improving nutrient uptake (Gonzalez-Guerrero et al., 2005). Down-regulation during AM fungal colonization and up-regulation by zinc fertilization of a zinc transporter gene in *M. truncatula* (*MtZIP2*) suggests that it is not involved in AM improved zinc uptake (Burleigh et al., 2003).

1.4 Arbuscular mycorrhiza-induced resistance

It has been shown in numerous studies, that AM fungi improve the tolerance of host plants against different adverse abiotic conditions such as cold, drought, salinity, and heavy metals (AL-Karaki, 2006; Porcel et al., 2006; Aroca et al., 2007; Hildebrandt et al., 2007; Evelin et al., 2009; Miransari, 2010). Different mechanisms have been discussed from direct impact of extraradical hyphae on soil conditions (Ryan and Graham, 2004; Rillig, 2004; Rillig and Mummey, 2006; Auge et al., 2007; Rillig et al., 2010) to indirect influences on plant physiology (Lanfranco et al., 2002; Aroca et al., 2008; Ruiz-Lozano et al., 2010, 2012). Compared with the relatively limited number of abiotic stress factors, biotic stress can have a broad variation due to numerous different macro- and microorganisms that can attack the plant. Starting in the 1970s, the protection of plant roots against pathogens by arbuscular mycorrhiza has been reported (Dehne and Schönbeck, 1975; Schönbeck and Dehne, 1977). This protection covers diseases that are induced by different soil-borne pathogens including fungi, oomycetes, bacteria and nematodes (Azcon-Aguilar and Barea, 1996; Whipps, 2004; Pozo et al., 2010). Besides locally induced resistance in plant roots, arbuscular mycorrhiza can induce systemic resistance in non-colonized regions of a mycorrhizal root and also in aerial parts (Pozo and Azcon-Aguilar, 2007). Mycorrhiza-induced resistance (MIR) in aerial parts is, however, not always supported. Depending on the type of pathogen, its development in the shoot can be also enhanced in mycorrhizal plants (Borowicz, 2001). It has been often reported that induced resistance of plants needs a pre-established mycorrhiza, but there is also evidence that co-inoculation of AM fungi and pathogens can result in successful protection (Slezack et al., 2000; Thygesen et al., 2004; Khaosaad et al., 2007). In addition, the outcome of the interaction depends on the choice of the AM fungal isolate (Pozo et al., 1999, 2009; Sikes et al., 2009; Hayek et al., 2012; Veresoglou and Rillig, 2012). In general, species from the family Glomeraceae are better protecting plants against root pathogens than those belonging to the Gigasporaceae (Maherali and Klironomos, 2007).

Different mechanisms are proposed to explain mycorrhiza-induced resistance (MIR) against biotic stress. This includes improvement of plant nutrient status, competition between AM fungi and pathogens, modification of root biomass and structure, changes of rhizosphere microbial composition and activation and priming of the plant immune system (Azcón-Aguilar and Barea, 1996; Pozo and Azcon-Aguilar, 2007; Wehner et al., 2010; Jung et al., 2012).

Mycorrhizal plants usually exhibit a better growth which can be considered as a stronger capability to resist the attack of pathogens. In several reports it was discussed that increased root functions of mycorrhiza alleviate the negative effect of root pathogens increasing at least the tolerance of the plants to pathogen infestation (Azcón-Aguilar and Barea, 1996). Additional applied phosphate, however, did not result in reduced symptoms indicating that the mycorrhiza-improved uptake of this mineral nutrient is not the reason for AM increased tolerance (Fritz et al., 2006). Concerning resistance, it could be argued that plants with AM fungal-improved nutrient status could invest more in resistance. Such a mechanism was discussed to be the basis for the observation that mycorrhizal plants attacked by a caterpillar showed no growth promotion anymore, but limited herbivore performance (Kempel et al., 2010).

The carbon assimilated by photosynthesis is important for the survival of the plant, the AM fungus and for the pathogen. Within mycorrhiza, it is suggested that plants control AM fungal colonization via the control of carbon fluxes (Catford et al., 2003; Vierheilig, 2004). On the other hand, pathogens are able to manipulate these fluxes to ensure their spread inside plant tissues (Biemelt and Sonnewald, 2006; Bolton, 2009). Evidence for carbon competition could be the mutual reduced energy status of *F. mosseae* (former *G. mosseae*) and *Aphanomyces euteiches* in pea root (Larsen and Bødker, 2001).

In addition to competition for carbon, AM fungi and pathogens might be also competing for space. In an established mycorrhiza, the pathogen was not only excluded from root cells containing arbuscules, AM fungal-colonized parts of the root also showed a limited number of pathogen infection sites (Cordier et al., 1998; Vigo et al., 2000). Following this line of arguments, one could explain the better MIR performance of Glomeraceae by their intensive spread in the roots, but a positive correlation between colonization capacity and pathogen resistance could not be observed (Powell et al., 2009).

It is well documented that AM fungal colonization induces remarkable changes in root architecture (Hetrick, 1991). Mycorrhizal roots show reduced numbers of root hairs (Hetrick, 1991), but in most studies branching was stimulated by AM fungi (Tisserant et al., 1996; Paszkowski et al., 2002; Gamalero et al., 2004; Oláh et al., 2005; Orfanoudakis et al., 2010). These changes support mycorrhizal colonization due to the preference of AM fungi for actively growing tissues and cortex cells (Gutjahr et al., 2009). Increased root branching,

however, would increase susceptibility for pathogens and can be therefore not the reason for MIR (Norman et al., 1996; Sikes et al., 2009).

Microbial communities in the rhizosphere can be quantitatively and qualitatively changed by AM fungi via direct interactions and via mycorrhizal impact on root physiology, structure and exudation (Andrade et al., 1997; Artursson et al., 2006; Toljander et al., 2007; Badri and Vivanco, 2009). Change of the microbial community of mycorrhizal roots improved the resistance against root pathogens (Citernesi et al., 1996; Filion et al., 1999; Larsen et al., 2003; Selim et al., 2005; Li et al., 2007; Cruz et al., 2008). Among the different species that were identified in the rhizosphere having antagonistic effect against pathogens, some promote AM fungal colonization, and inhibit the growth and mobility of pathogens (Citernesi et al., 1996; Budi et al., 1999; Larsen et al., 2003; Li et al., 2007), while others improve plant growth and nutrient uptake (Artursson et al., 2006; Bharadwaj et al., 2008). The antibiotic compounds such as the heterocyclic fungicide phenazine and 2, 4-diacetylphlotoglucinal produced by rhizosphere microbes can directly inhibit the development of pathogens as well as induce systemic resistance of host plant (Berea et al., 1998; Iavicoli et al., 2003; Ryong et al., 2007; Siasou et al., 2009; Bharadwaj et al., 2008, 2012).

Plants developed sophisticated defense reactions that can be divided into a primary and a secondary immune system against pathogens (Panwar et al., 2010). The primary immune system is mediated by the interaction either of pathogen associated molecular patterns (PAMPS) and the corresponding plant receptors (PAMP-triggered immunity) or of effectors encoded by pathogen avirulence gene loci and the products of plant disease resistance genes (Effector-triggered immunity) (Jones and Dangl, 2006). The secondary immunity comprises pre-induced resistance reactions. It can be generally divided into systemic acquired resistance (SAR), where local infection by a pathogen leads to a systemic response prevents secondary infections from related and unrelated attackers, and induced systemic resistance (ISR) where the shoot gets a certain level of immunity after the root has been challenged by non-pathogenic microbes (van Loop et al., 1998; Durrant and Dong, 2004). SAR is mostly mediated by the salicylic acid (SA) signaling pathway and directed against biotrophs while jasmonic acid (JA) and ethylene (ET) are the main controllers of ISR against necrotrophs (Pieterse et al., 2009).

Induced resistance in the secondary immune response is not so much accompanied by directly activated defense reactions. A more common feature is a sensitized immune system, which boosts faster and stronger upon pathogen attack. This phenomenon is known as 'priming' (Conrath et al., 2006; Goellner and Conrath, 2008). Compared with direct defense activation, priming is an energy-saving strategy for plants in absence of a pathogen (Conrath et al., 2006). Defense priming is mainly known from ISR, but can be triggered not only by plant beneficial organisms, but also by pathogens and herbivores, and also by particular endogenous plant compounds (Goellner and Conrath, 2008; van Wees et al., 2008; Pozo et al., 2008; Pastor et al., 2012).

Because phytohormones are also important players in mycorrhizal interactions (Hause et al., 2007), interference between AM fungal colonization and the plant immune system has been suggested (Pieterse et al., 2012). Plant defense reactions were observed in mycorrhizal roots since a long time, but in most studies, the reaction was localized in arbusculated cells and defense gene products accumulated only to a low extent compared with plant-pathogen interactions (Gianinazzi-Pearson et al., 1996; García-Garrido and Ocampo, 2002). These defense reactions may control the development of AM fungi inside plant roots, because the obligate biotrophic character of AM fungi shares similarities with biotrophic pathogens. Interestingly, plant gene expression patterns in mycorrhizal rice roots showed twice as much overlaps with roots infected by a hemibiotroph than with roots infected by a necrotroph (Güimil et al., 2005). SA is the regulator of plant defense against biotrophs (Glazebrook, 2005) and has a negative effect on AM development (Blilou et al., 1999). In order to develop a compatible symbiotic interaction, AM fungi suppress certain SA-mediated defense responses (Kapulnik et al., 1996). In contrast, genes encoding enzymes involved in JA- biosynthesis are expressed at later stages of the symbiosis, the expression is localized in arbuscule-containing cells, and endogenous JA levels are elevated in fully established AM (Hause et al., 2002; Isayenkov et al., 2005). Because JA and SA signaling pathways are interconnected and often antagonistic (Pieterse et al., 2009), it seems as if AM suppress SA-regulated defense against biotrophic pathogens but stimulate JA-regulated defense against necrotrophic pathogens and JA-sensitive insects (Pozo and Azcon-Aguilar, 2007; Jung et al., 2012).

Based on similar plant responses to mycorrhization and to exogenous JA application and based on the fact that mycorrhizal-primed defense reactions were observed in plant roots and systemically in shoots, it is currently assumed that MIR depends mainly on the JA signaling

pathway and induces similar resistance as the rhizobacteria-mediated ISR (Pozo and Azcon-Aguilar, 2007; Jung et al., 2012). The other mechanisms discussed above might also play a role while their contribution might differ. They could be operative at the same time (Pozo and Azcon-Aguilar, 2007) or might act consecutively (Cameron et al., 2013).

1.5 The model plant *Medicago truncatula*

Since the 1990s, *M truncatula* (barrel medic) was chosen as a model plant to facilitate research on the legume-rhizobia symbiosis (Barker et al., 1990). Legumes, second to grasses, comprise one of the most important agricultural plant families. Besides direct use as protein source for humans and animals, legumes also play an important role in maintaining ecosystem functioning by bacterial nitrogen fixation in their nodules which reduces the application of chemical fertilizers and the subsequent pollution of ground water and atmosphere(Crews and Peoples, 2004). Legumes exhibit great economic importance. Soybean and peanut e.g. account for more than 30% of oil production of plant origin (Graham and Vance, 2003). The choice of *M. truncatula* originated from the Mediterranean region was based on its relative small genome (about 500 Mbp), its short generation time (3-4 Month), its self-pollination, and its amenability of genetic transformation (Cook, 1999).

Within legumes, most plants have large, complex genomes. For instance the pea genome is about 4000 Mbp. Although the genome of *M. truncatula* is small (2n=16), a high level of microsynteny was found to larger genomes including pea, alfalfa, and soybean (Gualtieri et al., 2002; Cannon et al., 2003; Young et al., 2003; Yan et al., 2004). In the *M. truncatula* consortium, great progress has been made in generating expressed sequence tags (ESTs) and genome sequences. So far, more than 200,000 ESTs have been generated and made public available, and a nearly whole genome sequence is available based on six bacterial artificial chromosomes and one fosmid library (Young et al., 2011). In order to test the function of *M. truncatula* genes, different systemic approaches have been followed including insertion mutagenesis (T-DNA, transposons and retrotransposons), fast neutron bombardment (FNB), targeting-induced local lesion in genome (TILLING), and RNA interference (RNAi) (**Table 1.1**) (Trieu et al., 2000; d'Erfurth et al., 2003; Tadege et al., 2005, 2008; Stark et al., 2006; Le Signor et al., 2009; Limpens et al., 2004). Among these methods, insertional mutagenesis has the advantage of tagging mutant with known inserted sequences (Pislariu et al., 2012). A large number of *M. truncatula* mutants were generated using the tobacco *Tnt1* retrotransposon

which uses a 'copy and paste' mechanism, has seed-to-seed stability, and is efficiently covering the whole genome (d'Erfurth et al., 2003; Wessler, 2006; Tadege et al., 2008).

Unlike the classic model plant Arabidopsis (Brassicaceae), *M. truncatula* is able to develop two main root symbioses. Besides nodule formation with *Sinorhizobium meliloti,* the occurrence of arbuscular mycorrhizas in *M. truncatula* facilitates the research on this ancient symbiosis. Together with another legume model plant *Lotus japonicus*, the plant enabled the discovery of the common symbiosis pathway which evolved with the arbuscular mycorrhiza and was later recruited by the root nodule symbiosis (described above). The specific genes (*DMI1*, *DMI2*, *DMI3*, *IPD3,MtVAMP*, and *MtVPY*) involved in this pathway are discovered in *M. truncatula* (Parniske, 2008; Pumplin et al., 2010; Murray et al., 2011; Horváth et al., 2011; Ivanov et al., 2012). Apart from formation, genes that play important roles in nutrient transportation in arbuscular mycorrhizal symbiosis are also identified and functionally studied (Harrison et al., 2002; Krajinski et al., 2002; Hohnjec et al., 2003; Javot et al., 2007b; Gomez et al., 2009). Besides beneficial symbioses such as AM and RNS, plants in natural environment encounter pathogenic micro-organisms. *M. truncatula* is also used in different studies on interaction with pathogens such as fungi, oomycetes, bacteria, nematodes etc. (Colditz et al., 2004; Leitner et al., 2005; Tivoli et al., 2006; Liu et al., 2007; Vailleau et al., 2007; Ameline-Torregrosa et al., 2008; Jasiński et al., 2009). Different *M. truncatula* ecotypes and/or lines (Jemalong A17 and F83005.5) that are resistant against or susceptible for pathogens were also discovered (Vailleau et al., 2007; Ameline-Torregrosa et al., 2008; Djébali et al., 2009). Among the interaction of *M. truncatula* with pathogens, different mechanisms including specific defense gene expression, involvement of phytohormones, priming, and rapid accumulation of toxic secondary metabolites were proposed as basis for resistance or susceptibility (Gamas et al., 1998; Salzer et al., 2000; Torregrosa et al., 2004; Cluzet et al., 2004; Leitneret al., 2005; Colditz et al., 2005; Die et al., 2007; Naoumkina et al., 2007; Vailleau et al., 2007; Landgraf et al., 2012).

1.6 H^+-ATPases and MtHA1

H^+-ATPases belong to the superfamily of P-type ATPases that hydrolyze ATP and use the

Table 1.1 Comparison of different approaches for *M. truncatula* mutagenesis **(**adopted from Tagede et al., 2005**).**

Method	Type of mutation	Average lesion frequency	Major advantages	Major disadvantages	Ease of cloning genes by forward genetics	Ease of reverse genetic screening	Flanking sequence database
Retro-transposon or Tnt1 tagging	Gene disruption by insertion	~15 per genome	Knockout and tagging of mutated gene	Requirement for tissue culture and hence possibility of somaclonal variation; moderately difficult to generate mutant population	Easy	Easy	Yes
T-DNA tagging	Gene disruption by insertion	1~2 per genome	Knockout and tagging of mutated gene	Requirement for transformation and tissue culture and hence possibility of somaclonal variation; difficult to generate mutant population	Easy	Easy	Yes
EMS and TILLING	Point mutations	~ 1000 per genome	Allelic series for functional analysis; easy to generate mutant population	Preponderance of weak alleles	Difficult	Moderate	No
Fast neutron mutagenesis	Deletion mutations	Unknown	Knockout and deletion marker of mutated gene; easy to generate mutant population	Low frequency of allelic variation other than loss of function	Moderate	Moderate	No
RNA-induced gene silencing	Transcript knock down	Not applicable	Rapid for phenotypes accessible via hairy roots or other transient assays	Requirement for tissue culture to generate stable transformants; unpredictable penetrance	Not applicable	Easy	No

Note: EMS, ethyl methane sulfonate; TILLING, target-induced local lesion in genome.

energy for the transport of cations and other compounds against concentration gradients. P-type ATPases are named because a phosphorylated aspartate is involved in the catalytic cycle. More than 40 genes are identified in this superfamily in Arabidopsis and rice (Baxter et al., 2003). P-type ATPase genes are divided into ten phylogenetic branches and six are found in plants. The six groups encode heavy metal pumps (P1B subfamily), ER-type Ca^{2+}-ATPases (P2A subfamily), auto-inhibited Ca^{2+}-ATPases (P2B subfamily), proton pumps (P3A subfamily), putative aminophospholipid ATPases (P4 subfamily), and P-type ATPases type 5 (P5 subfamily) (Axelsen et al., 2001). The proton pump branch including all H^+-ATPases can be further subdivide into five subfamilies and is the least divergent branch of the P-type ATPase superfamily (Duby and Boutry, 2009). H^+-ATPases that are close to the ancestral P-type ATPase form are only found in plants and fungi. They transport protons out of the plasma membrane creating a pH and potential difference for secondary transportation (**Figure 1.4**) (Duby and Boutry, 2009). A similar membrane potential is partially established by Na^+/K^+-ATPase in animals.

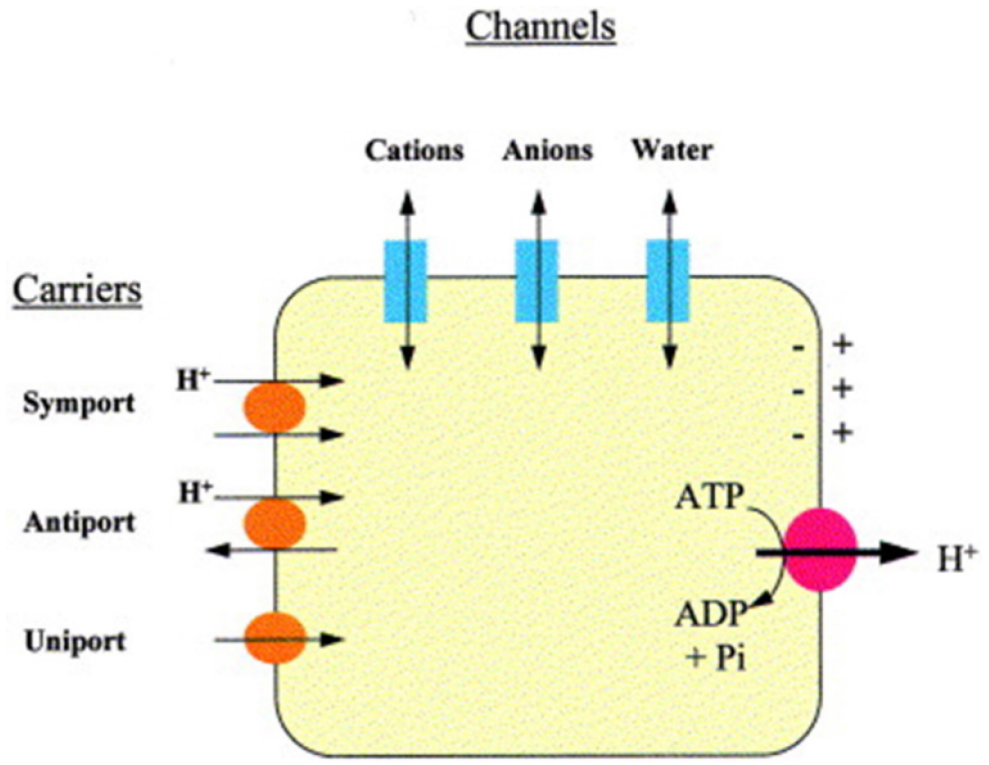

Figure 1.4 Primary and secondary transports across the plasma membrane (adopted from Morsomme and Boutry, 2000).

H^+-ATPases have N- and C-terminal segments, which protrude into the cytoplasm, and ten transmembrane segments that form two loops. The classical P-type ATPase possesses so-called A-, M-, P-, N- and R-domains (**Figure 1.5**). The A-domain corresponds to the actuator

domain and consists of the N terminal segment and the small loop. The M-domain corresponds to the membrane segment. The P-domain is located in the large loop and contains the phosphorylation site, while the N-domain carries the nucleotide binding site. Finally, the R-domain consists of the C-terminal part of the protein and acts as an autoinhibitor (Morsomme and Boutry, 2000; Duby and Boutry, 2009). Like the other P-type ATPases form oligomeric complexes, the monomer catalytic subunit of H^+-ATPases (~100 kDa) can oligomerize to form dimeric and hexameric complexes (Palmgren, 2001; Kanczewska et al., 2005).

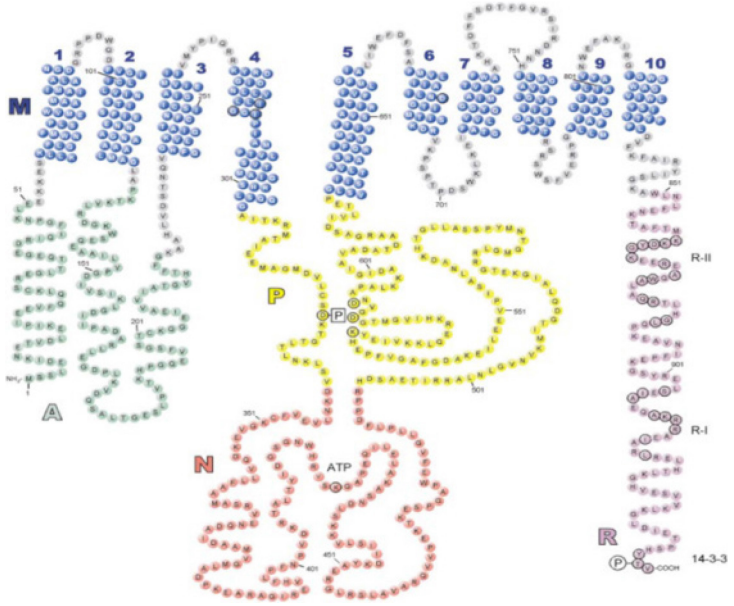

Figure 1.5 Domain composition of ATPases (adapted from Palmgren, 2001).

H^+-ATPases support a variety of biological functions and are encoded by multiple genes. By application of different methods including immunodetection, Northern blot analysis, *in situ* hybridization and GUS reporter gene constructs, differential expression of H^+-ATPase genes and their corresponding gene products was detected among different cell types, tissues, and organs (Morsomme and Boutry, 2000). Environmental factors such as salt stress, darkness, hormones like auxin and glucose can also regulate the expression of plant H^+-ATPase genes mainly on transcriptional level (Morsomme and Boutry, 2000; Portillo, 2000). Translational regulation of H^+-ATPases was first detected in fungi (Capieaux et al., 1989). In plants, the long 5'-untranslated region carrying small open reading frames determines the regulation on translational level, possibly by modulating reinitiation (Michelet et al., 1994; Lukaszewicz et al., 1998; Moriau et al., 1999; Morsomme and Boutry, 2000). In addition to transcriptional

and translational regulation, post-translational regulation also controls the activity of H^+-ATPases. As already mentioned, the C-terminal presents a negative autoregulatory domain in fungi and plants as its deletion increases H^+-ATPases activity. In addition, activity can be regulated by the amino-terminal region, the first and fourth transmembrane spans and by two cytoplasmic loops. The fungal toxin fusicoccin, different regulatory 14-3-3 proteins, the secondary messenger lysophosphatidylcholine, and the phosphorylation of particular residues by protein kinases all control its activity (Morsomme and Boutry, 2000; Portillo, 2000; Palmgren, 2001; Kanczewska et al., 2005; Duby and Boutry, 2009).

Plasma membrane H^+-ATPases are found in every investigated cell type, but their densities vary (Palmgren, 2001). The major role of plasma membrane H^+-ATPases is to facilitate secondary transportation. As the absorption of minerals from soil is against concentration gradients, H^+-ATPase activities provide the necessary electrochemical gradients for their active transport. In addition and mainly in fungi the uptake of organic compounds such as sugars is also enabled by H^+-ATPase activity (Morsomme and Boutry, 2000). H^+-ATPases also play a role in salt stress tolerance as Na^+/H^+ antiporters depend on the pH gradient generated by the proton pumps (Yamaguchi and Blumwald, 2005). The involvement of H^+-ATPases in the acid growth theory, the auxin induced tissue expansion and the fusicoccin-induced cell elongation makes H^+-ATPases an important player in plant growth process (Duby and Boutry, 2009). Another physiological function of H^+-ATPases is the control of stomata, as they are in charge of aperture regulation of guard cells (Palmgren, 2001; Duby and Boutry, 2009). H^+-ATPases are also involved in plant-pathogen interaction. They interact with the plant immune signaling protein RIN4 regulating stomatal apertures during bacterial invasion and its manipulation by pathogen effectors increases plant susceptibility (Elmore and Coaker, 2011).

As already mentioned, nutrient exchange is the main character of the mycorrhizal symbiosis and as it includes the transport of nutrients across membranes against concentration gradients, it was postulated that H^+-ATPases of both symbiotic partners must be involved (Smith and Smith, 1990).

In the mycorrhizal fungus *F. mosseae* (former *G. mosseae*), five fragments of genes encoding putative H^+-ATPases were originally identified among genomic sequences (Ferrol et al., 2000). The presence of two genes (*GmPMA1* and *GmHA5*) were later confirmed, and

expression analyses of these two genes implied the recruitment of particular H^+-ATPases during different developmental stages and their regulation by phosphate nutrition in soil and by sucrose availability inside the roots (Requena et al., 2003). Usage of specific P-type ATPase inhibitors indicated the involvement of ATPase in hyphal growth after spore germination and before hyphopodia formation on the root epidermis (Ramos et al., 2008). Identification of the H^+/NH_4^+ and H^+/P_i^+ symporters and pH dependent nitrogen and phosphate uptake indicated the function of H^+-ATPase also in nitrogen and phosphate uptake by mycorrhizal fungi (Pao et al., 1998; Harrison et al., 2002; Karandashov and Bucher, 2005; Ortiz-Ramirez et al., 2011; Pérez-Tienda et al., 2012).

On the plant side of the symbiosis, besides general mycorrhiza-regulated H^+-ATPase gene expression in leaves and roots (Ferrol et al., 2002), H^+-ATPase activity was detected specifically on the periarbuscular membrane formed around the AM fungal arbuscules (Marx et al., 1982; Gianinazzi-Pearson et al., 1991, 1995). Based on *in situ* hybridization and GUS reporter fusions, the particular members of the H^+-ATPase gene family were shown to be expressed in arbuscule-containing cells of tobacco, barrel medic and tomato (Gianinazzi-Pearson et al., 2000; Krajinski et al., 2002; Rosewarne et al., 2007).

1.7 *Aphanomyces euteiches*

Aphanomyces euteiches Drechs. is an oomycete pathogen causing severe root rot disease in legumes. This pathogen was first described by Jones and Drechsler (1925), and later discovered in America, Australia, Japan, New Zealand and Europe. It is considered as the most destructive disease of several legume crops including pea and alfalfa in areas with temperate or humid climate (Levenfors et al., 2003; Gaulin et al., 2007). It belongs to the phylum Oomycota, which is a large group and phylogenetically distinct from the Eumycota but more related to the diatoms, chromophyte algae and other heterokont protists (Gaulin et al., 2007). Among the Oomycota, the genus *Aphanomyces* belongs to the order Saprolegniales and contains plant-pathogenic species that are destructive to different hosts (Gaulin et al., 2008).

As a severe disease in pea, the first syndrome of *A. euteiches* infection is the softened and water-soaked root. Followed by rapid spread in the root cortex, the fine branches of rootlets are destroyed. Starting from the bottom of the shoot, leaves gradually turn yellow. Early infection can result inplants collapse and death while late infection affects only roots, while

plants still are able to produce pods (Gaulin et al., 2007). The life cycle of *A. euteiches* can be completed by sexual or asexual reproduction (**Figure 1.6**). Germinating oospores release more than 300 pyriform zoospores (Scott, 1961). After attached to host roots, zoospores are encysted by cyclic release and absorption of calcium ions (Deacon and Saxena, 1998). These cysts germinate and germinated hyphae penetrate root tips starting the infection. After entering plant roots, *A. euteiches* forms coenocytic hyphae and grows intercellular. During sexual reproduction, the formed haploid antheridia penetrate haploid oogonia with fertilization tubes and deliver male nuclei into oogonia. The fertilized spherical oogonia differentiate and finally form diploid oospores (Scott, 1961). During asexual reproduction, sporangia are formed by somatic hyphae and release zoospores to continue the life circle of *A. euteiches* (Hoch and Mitchell, 1972). In previous studies, different organs of *A. euteiches* including hyphae, zoospores and oospores were used as inoculum(Slezack et al., 1999; Thygesen et al., 2004; Colditz et al., 2004).

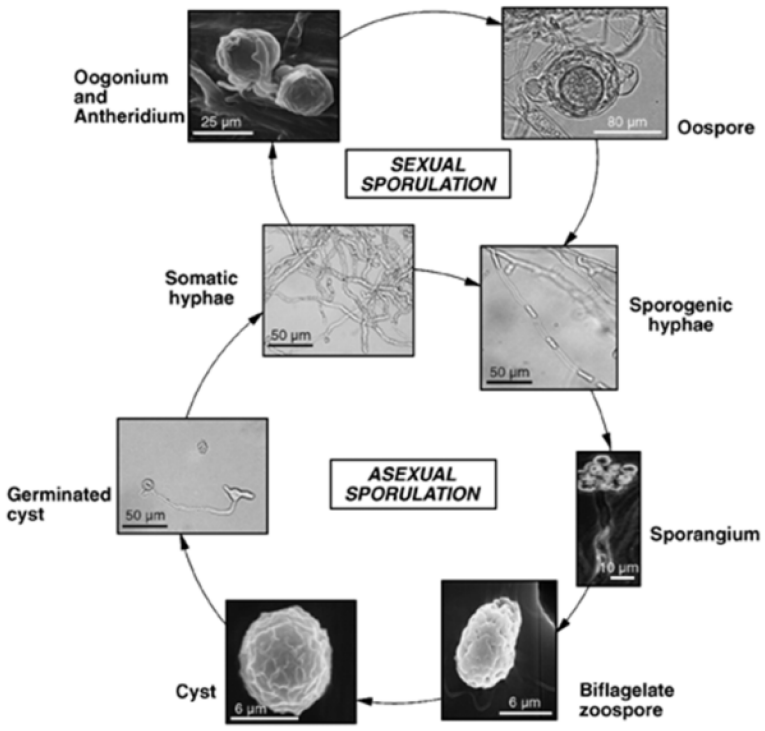

Figure 1.6 Life cycle of *A. euteiches*(adopted from Gaulin et al., 2007).

Different methods were used to identify and quantify *A. euteiches* in plant root systems. At the beginning, stained oospores in roots were counted (Morrison et al., 1971), but this method was time consuming and not suitable for analyzing large number of plants. The symptom based evaluation of infected roots is not reliable because the pathogen can invade root

systems without showing symptoms at early stages (Rao et al., 1985; Franken et al., 2007). Therefore, other methods for pathogen quantification were developed such as isozyme analysis (Larsson, 1994), enzymatic activity measurement by ELISA (Kjøller and Rosendahl, 1998; Slezack et al., 1999) and fatty acid profiling (Larsen et al., 2000; Larsen and Bødker, 2001). These methods were, however, either not specific for *A. euteiches* or only specific for one single isolate. Methods based on PCR are nowadays applied in pathogen detection and quantification as effective and time-saving methods (e.g. Vandemark et al., 2002; Vandemark and Barker, 2003; Vandemark and Grünwald, 2005), but have not been used for *A. euteiches*.

Control of *A. euteiches*-caused root rot disease is difficult as this pathogen can survive in soils for more than ten years, and oospores as surviving propagules remain viable even under extreme stress conditions (Gaulin et al., 2007). *A. euteiches* is able to fulfill its life cycle also in pasture and weeds as alternative hosts without obvious symptoms which prevents crop rotation as strategy to combat the pathogen (Chan and Close, 1987). Organic amendments such as the application of paper mill residuals was observed to reduce *A. euteiches* infection (Stone et al., 2003) and application of calcium amendments increases soil suppressiveness against *A. euteiches* (Heyman et al., 2007). Breeding for resistant pea cultivars was not successful. Only weak or partial resistant lines were identified and their resistance was not stable (Gaulin et al., 2007). By applying molecular genetics, quantitative trait loci (QTL) associated with partial resistance to *A. euteiches* were detected and used for breeding new at least more resistant pea cultivars (Pilet-Nayel et al., 2002, 2005). In a recent study using partial resistance lines of pea (PI 180693 and 553), 135 additive-effect QTLs corresponding to 23 genomic regions were identified (Hamon et al., 2011). In the legume plant alfalfa, showing also *A. euteiches*-caused root rot disease, the first resistant germplasm WAPH-1 was identified in 1989 (Grau, 1992), showing total resistance to *A. euteiches* race 1. Later WAPH-5 was reported to resist both, race 1 and race 2 (Malvick and Percissh, 1998; Malvick and Grau, 2001).

In order to bypass the limitation caused by large and complex genomes (pea and alfalfa) for identifying molecular markers and candidate genes, the model plant *M. truncatula* was introduced into research on legume-*A. euteiches* interactions (Nyamsuren et al., 2003; Franken et al., 2007). Its high level of microsynteny with other legume plants (see paragraph above) and its phenotypical variability makes*M. truncatula* extremely suitable for searching for different levels of resistance (Tivoli et al., 2006). Forty three *Medicago* accessions

selected from the French core collection representing 80% of the genetic variation of the genus were screened and their reactions towards *A. euteiches* infection varied from extremely susceptible to totally resistant (Moussart et al., 2007). Crossing of a resistant (DZA045.5) with a susceptible (FB83005.5) accession, one major locus (named *AER1*) was found on the terminal end of chromosome 3, where a resistance-gene-rich region is located. This locus explains 88% of the phenotypic variation (Pilet-Nayel et al., 2009). In another analysis using *M. truncatula* lines Jemalong A17 (partial resistant) and FB83005.5, a QTL named *prAe1* was also located on chromosome 3 (Djébali et al., 2009).

Application of different methods for the analysis of the pathosystem *M. truncatula* - *A. euteiches* revealed a number of mechanisms for this model interaction. Screening of a subtractive cDNA-library containing 560 expressed sequence tags (ESTs), a number of genes were identified which are induced in *M. truncatula* roots after *A. euteiches* infection (Nyamsuren et al., 2003). The hypothesis that the *PR10* gene family and the phytohormone abscisic acid (ABA) are involved in the defense reaction was later confirmed by proteome analysis (Colditz et al., 2004). Further comparative proteomics identified a number of additional proteins such as the proteasome alpha subunit in *M. truncatula* defense (Coditz et al., 2005). Silencing of PR10 protein expression revealed that a PR5 protein, which is normally inhibited by the PR10 protein, is an important player for resistance of *M. truncatula* against *A. euteiches* (Colditz et al., 2007). Other studies were based on comparing different *M. truncatula* lines. They revealed that the partial resistance of Jemalong A17 is based on more frequent pericycle root division, lignin deposition, accumulation of soluble phenolic compound and faster H_2O_2 accumulation (Djébali et al., 2009, 2011). In one experiment, elevated resistance against *A. euteiches* was achieved by systemically induced jasmonate acidproduction through repeated wounding on leaves indicating that this phytohormone is involved in *M. truncatula* defense reactions against the pathogen (Landgraf et al., 2012). Combined with the annotation of functions to genes identified in *A. euteiches* cDNA libraries will further extend our understanding about *A. euteiches* pathogenicity and the mechanisms of resistance or susceptibility (Gaulin et al., 2008; Badreddine et al., 2008).

The first evidence that arbuscular mycorrhizal fungi can act as bioprotectors against *A. euteiches* root rot of pea was reported by Rosendahl (1985). Further studies indicated that only a pre-established AM can efficiently induce resistance against *A. euteiches* while co-inoculation of AM fungi and pathogen does not (Rosendahl, 1985; Bødker et al., 1998;

Slezack et al., 1999, 2000; Bødker et al., 2002). In a field experiments, however, co-inoculation of an AM fungus with *A. euteiches* oospores reduced root rot in pea (Thygesen et al., 2004). After introduction of *M. truncatula* in *A. euteiches* studies (Nyamsuren et al., 2003), two mycorrhizal fungi were recorded aspotent protectors of roots against pathogen activity or oospore formation and spread (Colditz et al., 2005; Franken et al, 2007). Based on these analyses, the interaction between *M. truncatula*, *A. euteiches* and *F. mosseae* was established as experimental system to study mycorrhiza-induced resistance in legumes.

1.8 Dissertation objectives

M. truncatula is a model plant now widely applied in biological studies mainly concerning plant root symbioses. The choice of *M. truncatula* for the current Ph.D. dissertation was due to 1) the identification of a mycorrhiza-specific H^+-ATPase-encoding gene *MtHA1* and a line carrying a transposon insertion in this gene, 2) the availability of an experimental system to study mycorrhiza-induced resistance and 3) the recent availability of a nearly full genome sequence as valuable resource for molecular biology studies.

Arbuscular mycorrhizas can be considered as the oldest endosymbiosis formed between fungi from the phylum Glomeromycotawith plants (Parniske, 2008). The ecological and in future probably also economic importance of AM is mainly based on its capability to improve host plant nutrition and to protect roots against pathogens. Mechanisms behind biofertilization activity are getting started to be better understood based on the cloning and analysis of different mycorrhiza-specific plant and fungal nutrient transporters. The basis for bioprotection is, however, still elusive.

In the current PhD dissertation, the analysis of *MtHA1*, a plant proton pump-encoding gene putatively providing the electrochemical gradient necessary for nutrient transport should contribute to the understanding of the mutualistic nutrient exchange and its role in maintaining the mycorrhizal symbiotic relationship. In order to achieve this goal, particular genotypes of *M. truncatula* were inoculated with different isolates of arbuscular mycorrhizal fungi or with rhizobia under controlled environmental and fertilizer conditions. Growth of both plant and fungal partner of the symbiosis were monitored and the expression of marker genes for arbuscule functioning was analyzed.

For the purpose of better understanding arbuscular mycorrhiza-induced resistance, local and systemic interactions of *F. mosseae* and the oomycete pathogen *A. euteiches* in *M. truncatula* roots were evaluated and the expression of genes encoding for pathogenesis-related proteins and for enzymes involved in the biosynthesis of phytohormones or flavonoids was assessed.

In this context,

1 Effect of a *Tnt1* insertion in exon8 of *MtHA1* on the development of the arbuscular mycorrhizal symbiosis and on phosphate uptake was analyzed (Result **3.1**).

2 Effect of a *Tnt1* insertion in exon8 of *MtHA1* on mycorrhizal functions with two AM fungal species under different phosphate concentrations was compared (Result **3.2**).

3 Effect of a *Tnt1* insertion in exon8 of *MtHA1* on *Paris*-type mycorrhization with strong carbon sink was analyzed (Result **3.3**).

4 Effect of a *Tnt1* insertion in exon8 of *MtHA1* on nodule formation and function was analyzed (Result **3.4**).

5 Effect of a *Tnt1* insertion in *MtHA1* exon7 was analyzed (Result **3.5**).

6 Effect of jasmonates in arbuscular mycorrhizal locally induced bioprotection against *A. euteiches* was analyzed (Result **3.6**; only part of the published results were achieved by the candidate).

7 Effect of functional arbuscules for *A. euteiches* bioprotection was analyzed (Result **3.7**).

8 Effect of local and systemic interaction of arbuscular mycorrhiza and *A. euteiches* in *M. truncatula* roots was analyzed (Result **3.8**).

2 Materials and Methods

2.1 Biological material

Medicago truncatula

The legume *Medicago truncatula* was selected as a model plant due to its small, diploid genome, self-fertile nature, prolific seed production and rapid generation time (Cook, 1999). For this work, two different lines were used (Jemalong A17 and genotype R108). Seeds of *M. truncatula* Jemalong A17 were provided by Professor Philipp Franken (IGZ, Grossbeeren, Germany). Seeds of the *MtHA1exon8::Tnt1*homozygous mutant and the corresponding *MtHA1* wild type line and of the*MtHA1 exon7*::*Tnt1* heterozygous line were provided by Professor Franziska Krajinski (MPI-MP, Golm, Germany). The mutant lines were generated by transposon insertion of *M. truncatula*genotype R108 (Tadege et al., 2008).

AM fungi

Three species (*Glomus mosseae*, *Glomus intraradices* and *Gigaspora rosae*) that belong to two orders (Glomerales and Diversisporales) of Glomeromycota were selected for this work. Commercial inocula (BIORIZE, Dijon, France) of *Glomus mosseae* (Nicolson & Gerd.) Gerd. & Trappe BEG12 and *Glomus intraradices* Schenck & Smith were used. *Gigaspora rosea* Nicolson & Schenck BEG9 was provided by Dr. Silvio Gianinazzi (INRA, Dijon, France). Because *G. mosseae* is renamed to *Funneliformis mosseae* and *G. intraradices* to *Rhizophagus irregularis* by Schüssler and Walker (2010),the new fungal names are used.

Aphanomyces euteiches

Aphanomyces euteiches Drechs. strain ATCC 201684 (Rosendahl, Department of Mycology, Botanical Institute, University of Copenhagen, Denmark) that belongs to the Oomycotawas provided by Professor Franziska Krajinski (MPI-MP, Golm, Germany).

Rhizobium

Sinorhizobium meliloti was provided by Professor Franziska Krajinski (MPI-MP, Golm, Germany).

2.2 Seed germination

Seeds of *M. truncatula* were first surface sterilized by 10 min treatment with concentrated sulfuric acid, washed 3 times with distilled water and incubated in 3% (v/v) sodium

hypochlorite solution for 3 minutes. After surface sterilization, seeds were washed three times with distilled water. Sterilized seeds were then placed on moist filter papers in petri dishes. The germination last for 5 days with the first 3 days at 4^{o}C and the last two days at room temperature in darkness.

2.3 Split root formation

Root tips of germinated *M. truncatula* seeds were cut by a sterile scalpel. The seedling was then grown on M-medium (Bécard and Fortin, 1988) for one week until they develop some roots. Then the seedlings were transplanted into pots containing substrate (sand: vermiculite= 1:1) to grow enough roots for split-root experiments.

2.4 Inoculation of plants by microorganisms

AM fungi

Inoculation of AM fungi was conducted when the seedling was transplanting to pots (700 ml) containing a mixture of sand and vermiculite at a 1:1 ratio (v/v). Commercial inoculum was mixed with growth substrate at a ratio of 1:10 (v/v) for *F. mosseae* and *R. irregularis*, and 1:20 (v/v) for *G. rosea*. The non-mycorrhizal treatments were mock inoculated with autoclaved inoculumplus filter drains (Schwarz et al. 2011).

Aphanomyces euteiches

The cultivation and production of *A. euteiches* zoospores was performed using a modified method described by Colditz et al. (2005). *A. euteiches* was propagated by transfer of 1 cm^2 agar pieces to plates containing 17 g/l corn-meal agar (Carl-Roth, Karlsruhe, Germany) followed by cultivation in darkness at room temperature. When the entire surface of the plate was covered by mycelium, the agar was cut up into 2 cm^2 pieces and 3-4 pieces were transferred to a new petri dish. After adding maltose peptone broth (MPB; 3 g/l maltose, 1 g/l peptone; SigmaAldrich, Taufkirchen, Germany), Petri dishes were incubated in darkness at room temperature for 4-7 days. MPB was then removed and the mycelium was rinsed by autoclaved deionized water 3 times for 45 min for each to remove all nutrients. Afterward, the mycelium was incubated in autoclaved lake water (Güterfelder See, Germany) for 2 days to produce zoospores, which were counted under light microscope using a Burker chamber.

10^5zoospores were applied to seedling stem basis of the inoculated treatments. The control treatment was mock inoculated with autoclaved lake water.

Rhizobium

One colony of *S. meliloti* which grow on TY agar plates (5 g tryptone peptone, 3 g yeast extract, 0.0147 g $CaCl_2$ and 30 g agar in 1 liter; Carl-Roth) was picked and grown in 20 ml TY liquid medium (TY medium without agar) at 28°C, 200 rpm. After 24 hours growth, the TY liquid medium was diluted within 1 liter Hoaglands' solution (2 mM PO_4^-, without N; protocol see below). Twenty ml of this solution was applied to seedling stem basis of the inoculated treatments. The non-inoculated treatment was mock inoculated with 20 ml mixture of 1 liter Hoaglands' solution and 20 ml TY liquid medium.

2.5 Growth condition and fertilization

Each treatment consists of 4 biological replicates. Each biological replicate was one pot containing 700 ml substrate (sand: vermiculite=1:1, v/v) and 3 seedlings. Pots were arranged in a randomized block design. Plants were grown under constant condition in a greenhouse (220 µE m^{-2} s^{-1} for 16 h; 22°C in light and 18°C in darkness; 65% humidity) and fertilized with 10 ml Hoaglands' solution two times a week. Hoaglands' solution containing three phosphate (KH_2PO_4) concentrations (0.01 mM, 0.1 mM and 1 mM) or no nitrogen nutrients (replace $NO_3^-$with Cl^- and keep the concentration of corresponding cations) were used (Hoagland and Arnon, 1938). Plants were additionally watered 3 times a week with 10 ml deionized water.

2.6 Pollen grain germination

Pollen grains were gathered from *M. truncatula* flowers that blossom for 3 to 5 days. Pollen grains were germinated in 100 µl liquid medium consisting of 18% sucrose, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.01%H_3BO_3, pH7.0. Pollen was germinated at room temperature in darkness for 30 min before spotted onto a glass slide and observed by light microscopy (Yokota et al., 2009). Pollen was scored as germinated when the pollen tube was longer than the diameter of the pollen grain.

2.7 Determinations of biomass and mycorrhization

Harvested plants were thoroughly washed by tap water, and subsequently dried by paper towels. Shoots and roots were weighted freshly. To determine dry matter, shoots were dried at 80^{o}C for 24 hours and then weighed again. Fresh roots were cut into 1 cm length segments and mixed thoroughly. One part of the root was used for mycorrhiza measurement, the remaining part of the root was immediately frozen in liquid N_2 and stored at -80°C for RNA extraction.

Fresh root samples from different treatments were stained with trypan blue according to the method of Phillips and Hayman (1970). Fifty 1-cm-long root segments were selected randomly from each of the stained samples. The segments were assessed by microscopy. According to Trouvelot et al. (1986) (**Figure 2.1**), infection frequency (F%), absolute mycorrhization intensity (M%), relative mycorrhization intensity (m%), relative arbuscule abundance (a%), and absolute arbuscule abundance (A%) were calculated using the "Mycocalc" programme (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

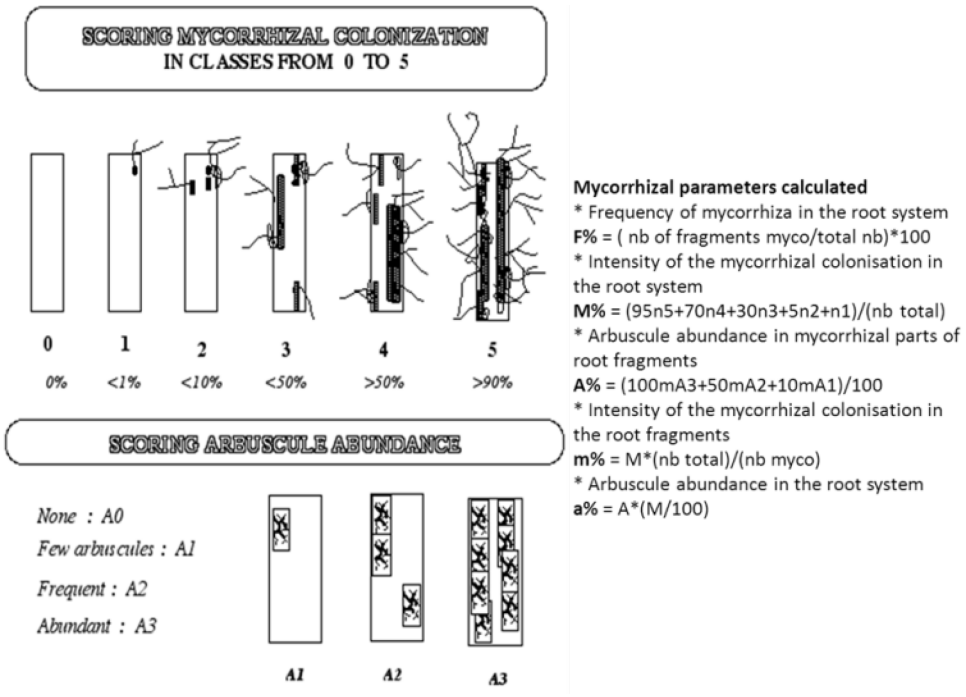

Figure 2.1 Scheme formycorrhiza evaluation (Trouvelot et al., 1986). Determination of mycorrhization by sorting, each root segments, mycorrhizal colonization in class from 0 to 5 and arbuscule abundance in 4 categories (A0, A1, A2 and A3).

The mycorrhizal growth dependency (MGD) was calculated based on the method from Smith et al. (2003) as followed:

$$MGD = 100 \times (DW_M - DW_{NM})/DW_M$$

DW_M: shoot dry weight of mycorrhizal plant

DW_{NM}: shoot dry weight of non-mycorrhizal plant

The mycorrhiza shoot/root ratio (MRI) increase was calculated as:

MRI =

Thermal Cycler (MWG Biotech, Ebersberg, Germany) under the following conditions: 95°C for 4 min, 35 x (94°C for 30 sec, annealing for 30 sec, 72°C for 30 sec),and a final 10 min extension at 72°C. After confirmation by 1.5 % agarose gelelectrophoresis, PCR products were purified on spin columns according to the manufacturer's protocol (Roche, Mannheim, Germany) and cloned into the pGEMTeasy vector (Promega, Mannheim, Germany). The sequencing and editing was performed by Eurofins MWG Operon (Ebersberg, Germany).

2.10 Quantitative Real-Time PCR and gene relative expression

Selected genes were quantified by Real-time PCR conducted on the 7500 fast real-time PCR system (Applied Biosystems, Foster city, USA) using SYBR green as fluorescent dye. Each reaction (10 µl) contained 5 µl SYBR green mix (SYBR green Low-ROX 2x Sensimix, Bioline, Luckenwalde, Germany), 0.4 µM of each tested primer (**Table 2.1**) and 1 µl of 1:10 diluted cDNA synthesized from 1 µg of total RNA. The amplification program was performed as follows: 95°C for 10 min, 40 cycles (95°C for 15 s, 60°C for 1 min). A melting curve (95°C for 15 s, 60°C for 1 min, 95°C for 15 s) was recorded at the end of every run to exclude primers generating non-specific PCR products (Ririe et al., 1997). Three biological replicates and three technical replicates were used for each treatment. The translational elongation factor-encoding gene *MtTEF1α* was used as constitutively expressed *M. truncatula* reference gene to normalize the expression level of the genes of interest. Relative gene expression levels were calculated as $2^{-\Delta CT}$($\Delta CT=CT_{gene}-CT_{mttef1\alpha}$).

Table 2.1 PCR primers, putatively encoded proteins of corresponding genes, their cellular functions, amplicon sizes and references

putatively encoded proteins	cell function	primer sequence (5'-3')	amplicon size (bp)	references
M. truncatula elongation factor *MtTEF1α*	protein synthesis	TGCTCGATTGCCACACCTCT TCTCCACAGCCTTGATGACACC	252	Wulf et al., 2003
F. mosseae elongation factor *FmTEF*	protein synthesis	AGTGCGGTGGTATTGACAAACG GCACAATCGGCCTGAGAAGTAC	246	Hilou et al., 2014
*A. euteiches*5.8s rRNA *AerRNA*	protein synthesis	TGTCTAGGCTCGCACATCGA AGTGCAATATGCGTTCAACGTTT	101	Hilou et al., 2014
AM specific phosphate transporter *MtPT4*	phosphate membrane transfer	TGCGATGACTTACTACTGG TGACGAATAAGTCCCATTGC	308	Grunwald et al., 2009
H^+-ATPase *MtHA1*: arbuscule specfic	proton pump	GACGGAAAATGGAGCGAGGAA ATCTGCAGGAATAATGTCACC	86	Krajinski et al., 2002
blue copper-binding protein *MtBCP1*	mediator of electron	TGGTATGTTTGTGGAGTTGC GATTTGAAAGAACTCATGCAC	372	Parádi et al., 2010
9-cis-epoxycarrotenoid dioxygenase *NCED*	abscisic acid biosynthesis	GGACGGTTTTATGCATGATCCT TGGCAAAGTCATGCATCATGAT	68	Planchet et al., 2011
1-aminocyclopropane-1-carboxylate synthase *ACS*	ethylene biosynthesis	CGACGAAATTTACTCAGGGACAG GCAGCGGCAACGACCAT	207	von Loonez al., 2006
allene oxide cyclase *AOC*	jasmonate biosynthesis	TTGAAGGGTGTTGCTGATTTGC GCAGCAGTAGATGGTTCAACATG	83	Isayenkov et al., 2005
isochorismate synthase *ICS*	salicylic acid biosynthesis	TATCGCCGTTTCCTTGTGCT AGTCCAAAGCTCCCTCTCCA	128	Wildermuth et al., 2001
pathogenesis-related protein 1 *PR-1*	anti-fungal protein	ATCCCCCAGAACATTGCTCG CCATCCAACACCACTACCCC	171	Nyamsuren et al. 2003

Table 2.10.1 continued

β-1,3-glucanase *PR-2*	anti-fungal protein	ACCCAGTTGGTGGCTCTTCTCA AGACGGCGGGTACGAAGTTCC	222	Nyamsuren et al., 2003
pathogenesis-related protein 4 *PR-4*	anti-fungal protein	ACCTGGGATGCCAACCAGCC GCCCAGCTGGTCCACAAAAGGC	73	Nyamsuren et al., 2003
thaumatin-like protein *PR-5*	anti-fungal protein	TTTGATGGCAGCGGTCGTGGC GTGGGACACCCCAACCGGTACA	79	Colditz et al., 2007
pathogenesis-related protein 10 *PR-10*	anti-fungal protein	GTGGCGCCGGAACCATCAAGA GTCTGGAAGGCCAACACCTCCA	137	Colditz et al., 2007
phenylalanine ammonium lyase *PAL*	flanovoid biosynthesis	AAATCGGCGGTGAGACACTT GCACCAAAACCGGTGGTAAC	178	Selim et al., 2010
chalcone synthase *CHS*	flanovoid biosynthesis	CAAAGGTGCTCGTGTGTTGG TGCCCCACAAGGCTATCAAG	93	Selim et al., 2010
chalcone reductase *CHR*	flanovoid biosynthesis	TGGTTGGAATGGGATCAGCCCC TCACCAAGAGCTTGTTCTGAGCCA	138	Colditz et al., 2005
isoflavonereductase *IFR*	flanovoid biosynthesis	CGAGGCAGTTGAGCCAGTTA GGCGTGGCAACAAAGGTAAG	103	Colditz et al., 2005
isoliquiritigenin (chalcone)-2-o- *COMT*	flanovoid biosynthesis	GCCTGCATCAACACAACACTCGG TCTCTCGGCGCCACCATCCT	133	Colditz et al., 2005

2.11 Measurements of phosphorus, nitrogen and soluble sugars

Phosphorus

Two methods were used for phosphorus extraction:

- 1. Dry ashing method: Samples of about 200 mg oven-dried shoots were dry-ashed at 500^{o}C, oxidized with 5 ml of 1:3 diluted HNO_3 on a hot plate, and suspended in 25 ml of 1:30 diluted HCL.
- 2. Microwave digestion method: Samples of about 200 mg oven-dried shoots were digested with 5 ml concentrated (65%) $HNO_3$and 2 ml 30% H_2O_2 at 200^{o}C for 15 minutes in a microwave (MARSXpress 250/50; CEM Corporation, North Carolina, USA) and suspended in 25 ml distilled water.

Phosphorus concentration was analyzed colorimetrically with a spectrophotometer (EPOS 5060, Eppendorf, Hamburg, Germany) at 436 nm wavelength after staining with ammonium-molybdate-vanadate solution (Gericke and Kurmies, 1952).

Nitrogen

Samples of 5 to 15 mg oven-dried shoots were analyzed after dry oxidation following the DUMAS method (Elementar vario EL; Hanau, Germany).

Soluble sugars

Root samples were frozen and ground to powder in liquid nitrogen. About 20 mg fresh weight of each samplewas transferred to an Eppendorf tube and 200 µl ethanol (100%, analysis grade) were added. The tube was incubated in a water bath at 78^{o}C for 20 minutes. After 5 minutes centrifugation at 14,000 rpm, the supernatant was transferred to a new Eppendorf tube. The pellet was re-suspended in 100 µl ethanol (50%) followed by incubation in a water bath at 78^{o}C for 20 minutes. After 5 minutes centrifugation at 14,000 rpm, the supernatant was transferred to a new tube. The extraction with 100 µl ethanol (50%) was repeated. The supernatants from 3 extractions were mixed, 10 µl was used for the Microplate reader assay (Biochrom anthos zenith 340; HNP Mikrosystem GmbH, Schwerin, Germany) described by Viola and Davies (1992).

2.12 Statistical analyses

Three-way, two-way or one-way analysis of variance (ANOVA) provided by the program package Statistica (version 9.1; StatSoft Inc., Tulsa, OK, USA) were used for statistical analyses. When significant interaction of factors was detected, post-hoc Fisher's-LSD was performed at P=0.05. Student t-test at P=0.05 was used to compare means of treatments.

3 Results

3.1Analysis of arbuscular mycorrhiza in the mutant *MtHA1exon8::Tnt1*

3.1.1 Introduction

A central characteristic for the mutualistic symbiosis formed between arbuscular mycorrhizal (AM) fungi and vascular plants is the exchange of nutrients. AM fungi provide plants with mineral nutrients especially phosphorus and receive carbohydrates from the plant (Smith and Read, 2008). Disturbance of this nutrient exchange affects formation and function of the symbiotic interaction. If for instance the AM specifically induced phosphate transporter gene *MtPT4*of*M. truncatula*, which is involved in phosphate transfer from the fungus towards the plant, was silenced, arbuscules died inside root cells before maturation and the fungus could not develop. This resulted in a complete break-down of the symbiosis (Javot et al., 2007b). In *L. japonicus,* knockdown of the AM-specific *LjPT3* gene led to a decrease of arbuscule numbers and significant reduction in phosphate uptake (Maeda et al., 2006). Similar results were also observed for *AsPT4* in *Astragalus sinicus* (Xie et al., 2013) and *OsPT11*(*ORYsa;PHT1;11*) in *Oryza sativa* (Yang et al., 2012). The unaffected mycorrhizal phenotype in *LePT4* silenced*Solanum lycopersicum* is due to functional redundancy where other mycorrhiza-regulated phosphate transporter genes ensure functioning of phosphate uptake via the mycorrhizal pathway (Nagy et al., 2005).Besides the influence of phosphate transfer, carbohydrate metabolism and transport also affects the symbiosis. In *M. truncatula*, knockdown of *MtSucS1* encoding a sucrose synthase resulted not only in reduced growth, delayed flowering and lower seed yield but also in down-regulation of AM marker genes and a reduced number of arbuscules with an increasing proportion of degenerated and dead arbuscules (Baier et al., 2010). In addition to the regulation by the plant, AM fungi also exert control on the mycorrhizal symbiosis. If *MST2* a symbiosis-induced gene encoding a monosaccharide transporter in *Glomus* sp. was deactivated by Host-Induced Gene Silencing, arbuscules inside root cell were either not fully developed or showed premature senescence and, the function of phosphate transport was almost completely abolished (Helber et al., 2011).

H^+-ATPases constitute a family of proton pumps driven by hydrolysis of ATPs and are found exclusively in the plasma membrane of plants and fungi (Duby and Boutry, 2009). By the activity of H^+-ATPases, an electrochemical gradient is formed that facilitates the active transport of solutes from the outside into the cell. In non mycorrhizal plant, plasma membrane

H^+-ATPases are located in the phloem, in stomatal guard cells, in root epidermis and root hairs, and in pollen tubes (Palmgren, 2001). In mycorrhizal plants, early studies confirmed the localization of H^+-ATPase at the periarbuscular membrane (Marx et al., 1982; Gianinazzi-Pearson et al., 1991). In plants, multigene families encode H^+-ATPases. Twelve genes were for example detected in *Arabidopsis thaliana* (Palmgren, 2001). In mycorrhizal roots of *M. truncatula*, three genes encoding H^+-ATPases were expressed. Besides the constitutive RNA accumulation of *MtHA2* and *MtHA3*, Northern blot analyses and *in situ* hybridization indicated that *MtHA1* is specifically expressed in mycorrhizal roots and the transcript is located in cells containing arbuscules (Krajinski et al., 2002).

Based on these findings it was hypothesized that *MtHA1*plays important role in mycorrhizal function (improving phosphate nutrient), and its encoded protein is providing the proton gradient which drives the phosphate uptake across the periarbuscular membrane. This hypothesis is tested by using a homozygous mutant of this gene (*MtHA1exon8::Tnt1*) which was found by F. Krajisnski in a population of *M. truncatula* lines with independent transposon insertions spread over the whole genome (Tadege et al., 2008). This mutant was compared with the corresponding wild type concerning plant growth, mycorrhization, phosphorus uptake, and gene expression.

3.1.2 Results

3.1.2.1 *MtHA1exon8::Tnt1* transcript structure

Germinated seedlings of the *MtHA1exon8::Tnt1* mutant (MUT) and the corresponding wild type (WT) were inoculated with *F. mosseae* when transplanted to soilless substrate and grown under low phosphate conditions (0.1 mM KH_2PO_4 in fertilizer solution). RNA was extracted six weeks after inoculation, cDNA was synthesized and fragments were amplified using primers located in exon 2 and exon 10 of the *MtHA1* gene (red arrow)(**Figure. 3.1-1A**). Gel electrophoresis of the PCR products showed that the fragment obtained from the mutant was shorter than the one from the wild type (**Figure. 3.1-1B**). The PCR product from the mutant was cloned and sequence analysis revealed that exon 8, where the transposon was located in the genome (data not shown), was missing resulting in a transcript with an internal deletion of 207 nucleotides.

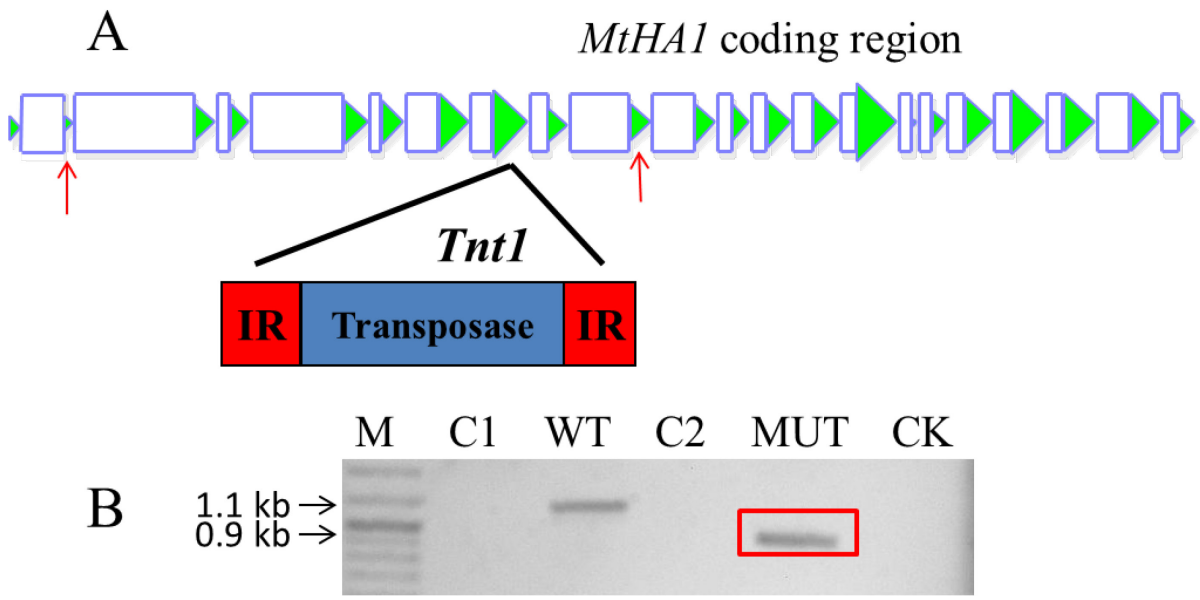

Figure 3.1-1 Structure and RT-PCR analysis of *MtHA1exon8::Tnt1*. RNA was extracted from root samples and used to synthesis template cDNA for PCR. Primers were located in exon 2 and exon 10 (red arrow A) (white squares represent introns while green arrows represent exons). Amplification products were separated by agarose gel electrophoresis (B). Sizes of PCR fragments are indicated. Mycorrhizal wild type (WT) and *MtHA1 exon8::Tnt1* mutant (MUT) showed amplified fragments while the mock-inoculated wild type (C1), mutant (C2) and negative control without template (CK) had no amplification production. The MUT amplification production (red square) was cloned and sequenced.

3.1.2.2 Plant growth

Mutant and wild type seedlings were inoculated with *F. mosseae* and grown together with mock-inoculated control plants under conditions as mentioned above. Plant growth parameters were measured two, four and six weeks after inoculation (**Table 3.1-1**). After two weeks of growth, plants were still small and no significant differences were observed in growth parameters between wild type and mutant plants. Shoot fresh and shoot dry weights of inoculated mutant plants were relatively larger than in the inoculated wild type, and resulted in a significant higher mycorrhiza growth dependency (MGD). The mycorrhiza root/shoot ratio increase (MRI) showed, however, no differencebetween wild type and mutant plants. At four weeks, all mycorrhizal plants showed increased biomasses of shoots compared to mock-inoculated controls and MGD values were 36% and 39%, respectively. At the same time roots of mutant plants were significantly larger than those of the wild type independently of

mycorrhization resulting in smaller shoot/root ratios: MRI of wild type plants was significantly higher than that of mutant plants. Six weeks after inoculation, mutant plants still had larger roots than wild type plants. Both genotypes retained the positive response in shoot biomasses to mycorrhizal colonization, but the effect was smaller in the mutant plants (28%) than in the wild type plants (54%). Compared among three harvest time points, the MRI of wild type plants gradually increased while in the mutant plants the effect increased at four weeks and then declined at six weeks. Analysis of variance indicated that growth time had a significant influence on total plant growth while mycorrhization only affected shoot and, genotype only affected root growth. No significant influence of factors alone was observed for MGD, but growth time and genotype interacted. Significant influence of factors and interaction between growth time and genotype was observed for MRI. Interaction of all parameters was also true for shoot fresh and dry weights and shoot/root ratio. Interaction concerning root growth was only detected between time and genotype.

3.1.2.3 Mycorrhiza formation

Mycorrhizal parameters of *M. truncatula* seedling were assessed in stained root samples every two weeks (**Table 3.1-2**). Mock-inoculated controls showed no infection (data not shown), while plants inoculated with *F. mosseae* harbored typical AM structures. After two weeks, all parameters of mycorrhization were small. Although higher values were observed in mutant plants, the difference was not significant due to the high standard deviations. At four weeks, the pattern changed and all parameters besides a% were significantly higher in wild type plants than in the mutant plants. Comparing data at two and four weeks after inoculation showed that all parameters were significantly increased in wild type plants, while no differences were observed in the mutant plants besides m%. Six weeks after inoculation, mycorrhizal structures in wild type plants were kept on the high level observed at four weeks. In contrast mutant plants maintained low values for most parameters. These differences during time were reflected by a significant interaction between the factors genotype and date of harvest.

After inoculation of *F. mosseae*, arbuscule morphology was monitored in wild type and mutant *M. truncatula* plants. As representatively shown in **Figure 3.1-2**, the arbuscules in wild type plants started to develop at two weeks, and fully branched arbuscules could be observed at fourand six weeks.

Table 3.1-1 Impact of mycorrhizationon plant growth parameters of wild type and *MtHA1 exon8::Tnt1* mutant plants. Average values of growth parameters ± standard deviation are shown. Three–way ANOVA indicated significant interaction between the factors date of harvest (time), genotype and mycorrhizal inoculation (myc) for shoot fresh weight (FW), shoot dry weight (DW) and shoot/root ratio. Two-way ANOVA indicated significant interaction between the factors of time and genotype for mycorrhiza shoot/root ratio increase (MRI) and mycorrhiza growth dependency (MGD). Values with different letter indicate significant differences (LSD-test $P = 0.05$, $n = 4$). WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; M, inoculated with *F. mosseae*; NM, without inoculation.

Harvest time	treatment	shoot FW (g)	root FW (g)	shoot DW (g)	shoot/root	MRI	MGD (%)
2 weeks	WT NM	0.16±0.07 f	0.06±0.02	0.01±0.006 f	2.51±0.31 ab	-	-
	WT M	0.18±0.06 f	0.08±0.02	0.01±0.004 f	2.32±0.19 bc	0.94±0.16 e	7±23 c
	MUT NM	0.18±0.05 f	0.08±0.03	0.01±0.002 f	2.30±0.31 bc	-	-
	MUT M	0.28±0.03 ef	0.11±0.02	0.02±0.002 ef	2.60±0.48 ab	1.15±0.27 e	48±4 ab
4 weeks	WT NM	0.38±0.12 e	0.29±0.11	0.04±0.013 e	1.37±0.24 de	-	-
	WT M	0.66±0.08 d	0.24±0.06	0.06±0.010 d	2.94±0.52 a	2.21±0.56 b	36±11 ab
	MUT NM	0.38±0.14 e	0.41±0.11	0.04±0.010 e	0.92±0.10 ef	-	-
	MUT M	0.59±0.08 d	0.40±0.07	0.06±0.009 d	1.49±0.07 d	1.63±0.19 c	39±9 ab
6 weeks	WT NM	0.64±0.12 d	0.82±0.20	0.09±0.015 c	0.80±0.06 f	-	-
	WT M	1.82±0.16 a	0.94±0.16	0.19±0.016 a	1.97±0.28 c	2.49±0.41 a	54±4 a
	MUT NM	0.89±0.05 c	1.40±0.20	0.12±0.009 b	0.65±0.10 f	-	-
	MUT M	1.23±0.13 b	1.40±0.17	0.17±0.023 a	0.88±0.02 f	1.39±0.23 d	28±8 b
time		s	s	s	s	s	ns
genotype		ns	s	ns	s	s	ns
myc		s	ns	s	s	na	na
time*genotype		s	s	ns	s	s	s
time*myc		s	ns	s	s	na	na
genotype*myc		s	ns	ns	s	na	na
time*genotype*myc		s	ns	s	s	na	na

Note: s, significant influence or interaction at $P = 0.05$, $n = 4$; ns, no significant difference at $P = 0.05$, $n = 4$; na, not applicable.

In mutant plants, the developing arbuscules at two weeks showed a similar phenotype as inwild type plants. At four and six weeks, however, only rarely branchedarbuscules could be observed in the mutantin instead of the fully branched arbuscules present in wild type roots.

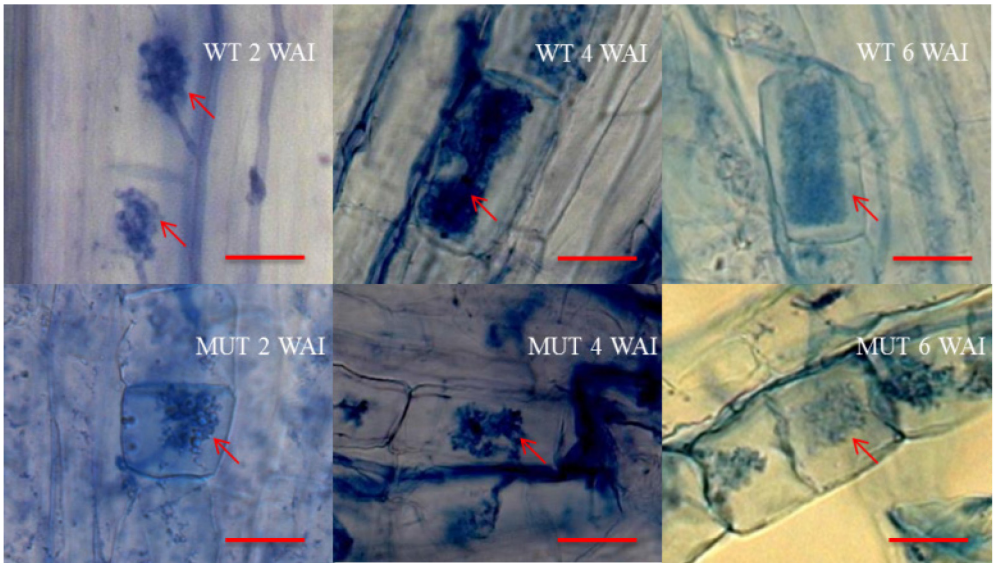

Figure 3.1-2 Arbuscules formed in *M. truncatula* root of wild type and *MtHA1exon8::Tnt1* mutant plants. Two weeks after inoculation of *F. mosseae*, only small (developing) arbuscules were observed in the wild type. Four weeks and six weeks later, fully branched arbuscules appeared in wild type plant roots. In mutant plant roots, the arbuscules maintained the rarely branched status at all-time points. WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; WAI, weeks after inoculation. Bars = 20 µm.

Table 3.1-2 Mycorrhizal parameters in wild type and *MtHA1 exon8::Tnt1* mutant plants. Average values of different parameters ± standard deviation are shown. In mock-inoculated samples, no mycorrhizal structures were observed. Two-way ANOVA revealed significant interaction between the factors genotype and harvest time.Values with different letter indicate significant differences (LSD-test *P*= 0.05, *n*=4). WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; M, inoculated with *F. mosseae*; F%, infection frequency; M%, absolute mycorrhization intensity; m%, relative mycorrhization intensity; a%, relative arbuscule abundance; A%, absolute arbuscule abundance.

Harvest time	treatment	F%	M%	m%	a%	A%
2 weeks	WT M	30.94±9.95 b	1.18±0.54 b	3.64±1.02 c	11.54±13.12	0.17±0.2 b
	MUT M	31.95±12.69 b	1.74±1.6 b	4.83±4.11 c	13.28±20.64	0.4±0.62 b
4 weeks	WT M	79.68±16.09 a	36.71±15.23 a	44.53±11.03 a	24.91±9.52	9.51±4.76 a
	MUT M	42.15±5.7 b	9.44±4.35 b	21.89±7.19 b	15.02±5.14	1.58±1.3 b
6 weeks	WT M	78.83±10.68 a	26.25±14.01 a	32.36±14.59 ab	49.38±12.03	13.73±8.69 a
	MUT M	45.58±9.23 b	10.82±3.79 b	23.3±5.56 b	20.29±4.48	2.22±0.89 b
time		s	s	s	s	s
genotype		s	s	s	s	s
time*genotype		s	s	s	ns	s

Note: s, significant influence or interaction at *P*=0.05, *n = 4*; ns, not significant at *P*=0.05, *n = 4*.

3.1.2.4 Phosphorus uptake

Phosphorus (P) concentrations were measured in shoots of mycorrhizal and mock-inoculated wild type and mutant plants at six weeks. Based on the data obtained and on the dry weight of shoots (**Table 3.1-2**), phosphorus uptake was calculated (**Figure 3.1-3**). All inoculated plants showed higher values for uptake than mock-inoculated controls. The effect was, however, partially inhibited in the mutant plants as the value was significant lower than in the corresponding wild type plants.

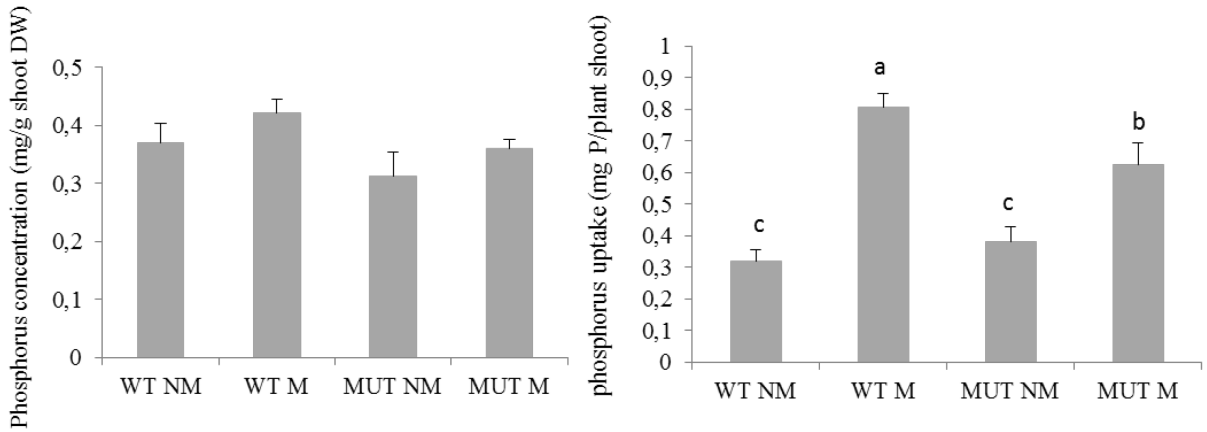

Figure 3.1-3 Impact of mycorrhization on phosphorusconcentration and uptake six weeks after inoculation. Uptake rates were calculated in *M. truncatula*mutant and wild type plants based on measurements of phosphorus concentrations and shoot dry weights (P uptake= P concentration × shoot dry weight). Shown are mean values and standard deviations. Two-way ANOVA showed significant interaction between factor genotype and mycorrhization for the parameter P uptake. Letters above columns indicate significant difference between treatments (LSD-test P= 0.05, n=4). WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; M, inoculated with *F.mosseae*; NM, without inoculation.

3.1.2.5 Relative gene expression

In order to test the function of the arbuscules in wild type and mutant plants, the expression of three *M. truncatula* genes were studied by quantitative real-time RT-PCR: *MtPT4* encodes a mycorrhiza-specific induced phosphate transporter which is located on the fine arbuscule branches (Harrison et al., 2002; Pumplin et al., 2012). The *MtHA1* gene is also mycorrhiza-specific expressed (Krajinski et al. 2002), but the protein has up to now not been localized. As the *Tnt1*retrotransposon is inserted in exon8 of the gene (**Figure 3.1-1A**), the primers for measuring RNA accumulation were located in the *MtHA1* exon6. Finally,the expression of the mycorrhiza-induced gene *MtBCP1* was analyzed. It encodes a blue copper-binding protein which is located at the trunks of the arbuscules, but not at the fine branches (Pumplin and Harrison 2009). At two weeks, *MtPT4* and *MtHA1* were induced by mycorrhization while

MtBCP1 showed no significant difference among treatments. When related to arbuscules numbers, the expression level of *MtPT4* and *MtHA1*seemed to be higher in wild type plant but this difference was not significant (**Figure 3.1-4**). At four and six weeks, all three genes were induced in wild type mycorrhiza as expected, but this induction was abolished in mutant plants. Whencorrelated with arbuscule number, the expressions differ between the two genotypesfor *MtPT4* and *MtBCP1*at four weeks, but not significantly. At six weeks, the difference between wild type and mutant plants was significant for *MtPT4* and *MtHA1*, but not for*MtBCP1*(**Figure 3.1-4**).

3.1.3 Discussions

The infection of rootby a mycorrhizal fungus is a continuously occurring process and develops with the root system as the fungus spreads outside to newly formed roots. In this way, mycorrhizal infection of different root parts occurs in an unsynchronized manner. Plants play an important role in regulation of the symbiosis and can control the infection level. Pre-inoculated plants for example can suppress subsequent infection by the same or by otherAM fungi (Vierheilig et al., 2000; Catford et al., 2003).In the current study, the development of *F. mosseae* in wild type plants followed the following pattern. After initial multiplepenetration observed at two weeks, the AM fungus rapidly spread inside the root cortex, and reached a relatively highinfection level at four and six weeks (F% in **Table. 3.1-2**). In mutant plants, besides the initially similar fungal penetration as in wild type plants, latter development was significantly reduced. As an obligate biotroph, AM fungus needs carbohydrates from host plants to develop intra- and extra-radically (Bago et al., 2002) while supplying mineral nutrient such as phosphate to the host (Karandashov and Bucher, 2005). In acompatible AM symbiosis, the spread of intraradical hyphae supports the development of extraradical hyphae which infect the newly formed plant roots (Smith and Read, 2008). In the current study, together with the limited intraradical hyphae spread (M% and m% in **Table 3.1-2**), it can be assumed that the development of extraradical hyphae was limited and resulted in a reduced subsequent infection at four and six weeks in *MtHA1* mutant plants. Combined with the importance of supplying phosphate for maintaining the AM symbiosis, it can be speculated that silencing of *MtHA1* limited the phosphate supply by the AM fungus and consequently reduced the carbohydrate transfer from the plant which is vital for intraradical and extraradical AM fungal development (Maeda et al., 2006; Javot et al., 2007a; Kier et al., 2011; Xie et al., 2012; Smith and Smith, 2012).

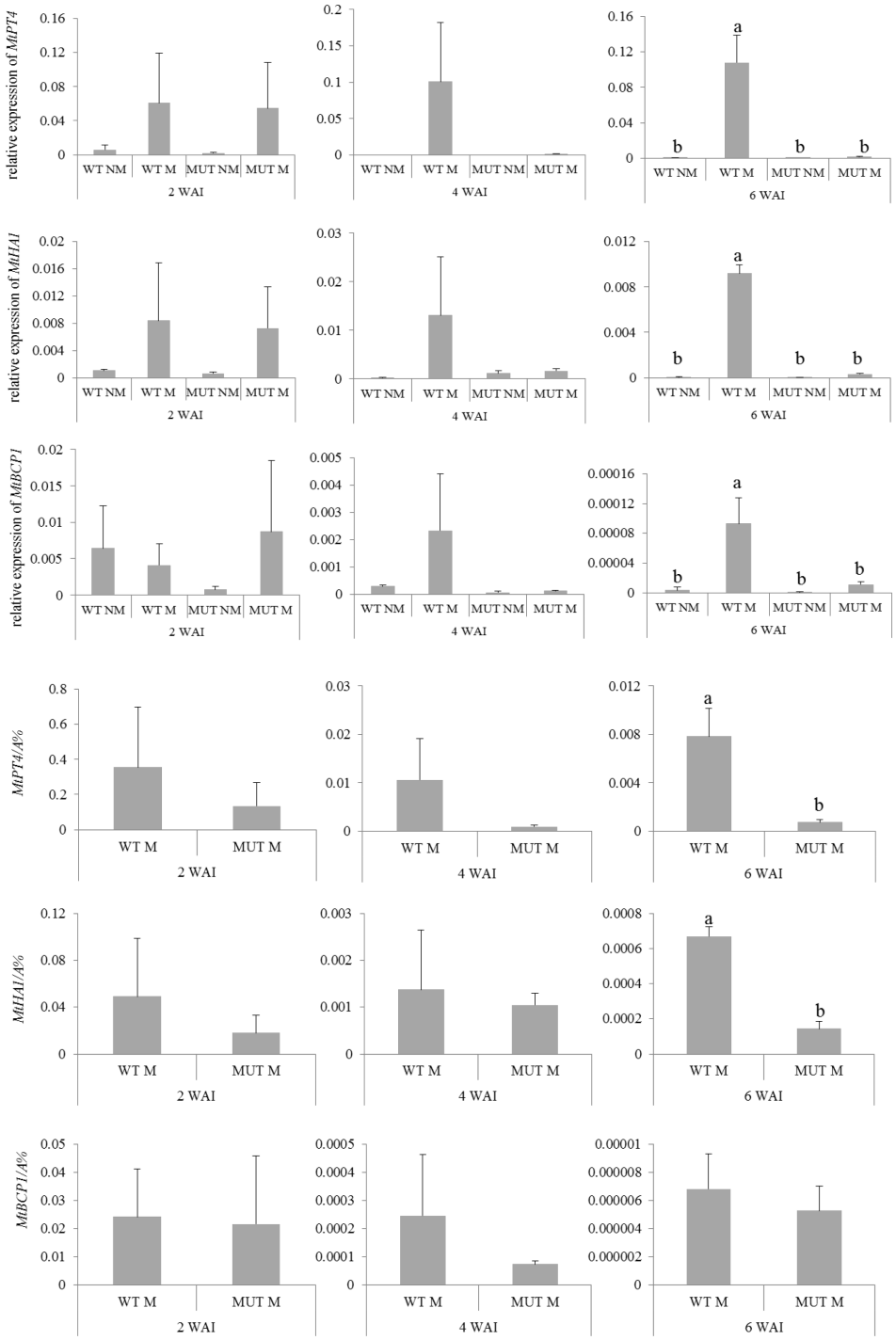

Figure 3.1-4 Gene expression levels of *MtPT4*, *MtHA1* and*MtBCP1*.RNA was extracted from roots of wild type and *MtHA1 exon8::Tnt1* mutant plantsinoculatedor not with*F. mosseae*. Values obtained for the three arbuscule-specific genes were normalized with the values of*MtTEF1α*as reference gene (upper graphs).Bottom graphs show the ratio between RNA accumulation levels and arbuscule numbers for the three genes in wild type and mutant plants.Two-way ANOVA revealed significant interaction between factors genotype and mycorrhization. Letters above columns indicate significant difference between treatments (LSD-test P= 0.05, n= 3). WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; M, inoculated with *F.mosseae*; NM, without inoculation; A%, absolute arbuscule abundance. Bars = standard deviations.

When the mycorrhizal fungus reaches inner cortex cell, fungal hyphae penetrate cell wall and branches repeatedly to produce the tree-shaped structure called arbuscule in the apoplast. The arbuscules are excluded from host cytoplasm by a host-derived periarbuscular membrane (Smith and Read, 2008). During hyphal branching, the formation of pre-branching cytoplasmic aggregations inanalogy to the prepenetration apparatus (PPA) issuggested to play a role in extending the periarbuscular membrane and defining the growth of the hyphal branches (Genre et al., 2008). As demonstrated by Pumplin and Harrison (2009), the periarbuscular membrane is composed of an "arbuscule branch domain" containingMtPT4 and an "arbuscule trunk domain" containingMtBCP1.According to Javot et al. (2007b), the process of hyphal branching is interrupted in *MtPT4* silenced plants andthe occurrence of septa indicates the senescence of arbuscules. Because the arbuscule branch domain is the site where MtPT4 is located, it can be assumed that the electrochemical gradient for driving the activity of the transporter is also needed here.In the *MtHA1* mutant of the current study the branching of the arbuscules was also interrupted but unlike in the *MtPT4* mutant, fungal spread was not totally abolished. The mycorrhizal phenotype assembles the results in silencing phosphate transporter in tomato, rice and *A. sinicus* (Nagy et al., 2005; Yang et al., 2012; Xie et al., 2013). Based on the importance of phosphate from AM fungus and the discovery of the so-called transcepter (AsPT1 and OsPT13), it is reasonable to hypothesize that the transcepter in the *MtHA1* mutant *M. truncatula* was functional and it maintained AM symbiosis (Javot et al., 2007b; Yang et al., 2012; Xie et al., 2013). Further evidences are needed to illustrate the identity of the transcepter in *M. truncatula* and its interaction with *MtHA1*.

The RNA accumulations of three genes expressed in arbuscule-containing cells were monitored in the current study at three time points. At two weeks, inoculation of *F. mosseae* induced the expression of *MtPT4* and *MtHA1* in both wild type and mutant plants. At this stage, the fungal hyphae just reach the inner cortical cells and start to develop arbuscules that showed no difference in wild type and mutant plants. This undifferentiated initial development was also observed in *MtPT4*–silenced plants (Javot et al., 2007b). During this stage, proteins such as MtPT4 and a mycorrhiza-specific ABC transporter stay in the potential cargo of VAMP72d/e vesiclesto be later integrated into the developing arbuscule branches (Harrison, 2012). In a similar way,MtHA1 could also be located in this cargo waiting for the secretion to form the arbuscule branch domain membrane. The feedback signaling from a non-functionalMtHA1has not been started and thereforeinduction of transcriptionis similar in

wild type and mutant plants. After this initial stage, the mutated MtHA1 protein, which either cannotbe localized in the developing periarbuscular membrane or is localized but shows no function, is not able to build up the electrochemical gradient across the periarbuscular membrane. The pH in the apoplast is not decreasing as can be seen in normally developed arbuscule-containing cells (Krajinski et al. submitted). This lack is sensitized by the plant cell and/or the fungal hyphae via an unknown mechanism (see also below) and preventssubsequent development of arbuscule branches as could be observed by microscopy (**Figure 3.1-2**). In consequence the further expression of the genes encoding proteins located in the arbuscule branches is repressed in the mutant plants at four and six weeks. This is reflected by the fact that not only the overall expression of the genes is reduced, but also the expression relative to arbuscule abundance (**Figure 3.1-4**).The *MtBCP1* gene encoding a copper binding protein has been found strongly correlated with AM fungal arbuscule formation (Parádi et al., 2010) and the protein is specifically localized at the membrane of the arbuscule trunk (Pumplin and Harrison, 2009).It is therefore not surprising that this gene is only similarly reduced in its expression as the number of arbuscules is reduced, but that the expression relative to arbuscule number did not change.

When the ability of a plant obtaining nutrients especially phosphate from its mycorrhizal fungal partner is reduced, the plant will restrict the development of the fungus inside the roots and this is probably due to auto-regulatory mechanisms to limit the costs for establishing the symbiosis (Catford et al., 2003). In *MtPT4*-silenced plant, diminished phosphate uptake accompanied by failure of the symbiosis suggested that receiving phosphate from the AM fungus is the basis for establishment of the symbiosis (Javot et al., 2007b). In the current study, phosphorus uptake was improved in plants colonized by *F. mosseae*, but this improvement was significantly suppressed in mutant plants. Unlike in mutants where the mycorrhizal induced phosphate transporter was silenced (Maeda et al., 2006; Javot et al., 2007b; Yang et al., 2012; Xie et al., 2013), still an increased phosphate uptake could be observed in mutant plants. This could indicate that either a limited amount of phosphate was delivered towards the plant via the mycorrhizal pathway especially in the beginning of the interaction where no obvious difference was observed between mycorrhizal and non-mycorrhizal plants or the direct uptake was enhanced in plants colonized by the fungus without establishing the arbuscule interface. One evidence of the AM stimulated direct phosphate acquisition is the identification of mycorrhizal-activated plant acid phosphatases,

which are in charge of hydrolyzing organic phosphate and salvaging phosphate esters leaked from root cells (Ezawa et al., 2005; Li et al., 2012).

In the current study, significant interaction between the factors genotype and mycorrhizal inoculation for the parameters shoot fresh weight and dry weight was detected. In the wild type, a mycorrhizal improved growth could be observed. The mycorrhizal growth dependency increased from 7% at two weeks to more than 50% at six weeks. The improved shoot growth can be attributed to enhanced phosphorus uptake by mycorrhizal fungi (Smith and Read, 1997).In mutant treatments, the growth improvement of mycorrhiza decreased with time, but still the plants inoculated with *F. mosseae* showed improved growth parameters, which indicated that the growth promoting effect was only partially suppressed. This was consistent with the experiment in which a phosphate transporter encoding gene (*LjPT3*) of *L. japonicus*was silenced but the AM fungus still improved the plant growth and the phosphate uptake when compared with the non-inoculated mutant plants (Maede et al., 2006). In *M. truncatula*, silencing of the AM specifically induced phosphate transporter gene (*MtPT4*) abolished phosphate uptake via mycorrhizal pathway, and poly-phosphate was piled up in arbuscules and intraradical hyphae (Javot et al., 2007b). The abolished phosphate uptake was also observed in rice and *A. sinicus* when the orthologues of *MtPT4* were silenced (Yang et al., 2012; Xie et al., 2013).

In summary, the data support the hypothesis that *MtHA1*plays an important role in mycorrhizalformation (arbuscule branching) and function (improving phosphate nutrient), while it is not certain that MtHA1 is providing the proton gradient which drives the phosphate uptake across the periarbuscular membrane. The function of *MtHA1* in phosphate uptake is, however, not as important as *MtPT4* in the mycorrhizal symbiosis. If phosphate uptake improvement is realized by other mechanisms as discussed above or if still transfer across a residual periarbuscular membrane takes place, has to be further investigated. The electrochemical gradient could be e.g. provided by the other plant proton pumping ATPases expressed in the root (Krajinski et al. 2002) or by proton pumps encoded by the fungus and expressed in the fungal arbuscules(Requena et al., 2003).

3.2 Effect of *Tnt1*insertion in *MtHA1* exon8on mycorrhization with two AM fungi at different phosphate fertilizer levels

3.2.1 Introductions

Phosphorus is a major nutrient and serves for a variety of structures like nucleic acids or phospholipids, and for different functions like energy metabolism, signal transduction cascades or enzyme regulation (Karandashov and Bucher, 2005). Although large amounts of phosphorus are presentin natural environments, most of it is in low soluble forms as phosphates of iron, aluminum and calcium. In plants, besides the direct phosphate uptake via root epidermis and root hairs, an alternative phosphate uptake pathway via mycorrhizal fungi exists that can bridge the depletion zone around the root (**Figure 3.2-1**) (Smith and Read 1997). Phosphate concentrations in the surrounding soil affect root colonization by AM fungi (Franken and Gnädinger, 1994). When plants grow under phosphate deficient conditions, roots are highly colonized, but with increasing phosphate concentrations, host plants seem to reduce mycorrhization (Breuillin et al., 2010; Branscheid et al., 2010). Fertilization with high amounts of phosphate does not only reduce the amount of colonization, but also repress the phosphate transporters of the mycorrhizal uptake pathway (Nagy et al., 2009). Nevertheless an experiment with isotope-labeled phosphate in a compartment system indicated that also under high phosphate concentration condition, AM fungi still supply host plants with this macronutrient (Smith et al., 2004).

In natural environments, one plant can be colonized by AM fungi from different taxa. Because plant growth response to different AM fungi varies, a functional diversity among plant-AM fungi symbioses was hypothesized (Franken and George, 2006; Smith et al., 2011). For example, symbioses formed with *F. mosseae* or *R. irregularis*, probably the most studied AM fungi, show different features. Under water deficiency, increase in drought tolerance by *F. mosseae* is much higher than by *R. irregularis* (Ruiz-Lozano et al., 1995). *F. mosseae* also reduced disease symptoms in tomato and petunia infected with *Phytophthora parasitica* or *Thielaviopsis basicola*, but *R. irregularis* did not (Pozo et al., 1999; Hayek et al., 2012). Compared with *R. irregularis*, *F. mosseae* shifted more the balance from arsenic to phosphate uptake in *M. truncatula* (Christophersen et al., 2012). *R. irregularis*, however, infected roots much faster than *F. mosseae* and this infection was much less impaired by high amounts of phosphate (Alkan et al., 2006). In extreme cases, *R. irregularis* was able to supply 100% of the phosphate demand of tomato plants (Smith et al., 2004). Transcriptional profiling of *M.*

truncatula roots inoculated with *F. mosseae* or *R. irregularis* indicated that 2.5% of allinvestigated genes were induced or repressed by both symbionts, but 10.6% were only affected by the one or the other mycorrhizal fungus (Hohnjec et al., 2005). Five phosphate transporter genes of the direct pathway are for example down-regulated by *R. irregularis*, but only two by *F. mosseae* (Grunwald et al., 2009).

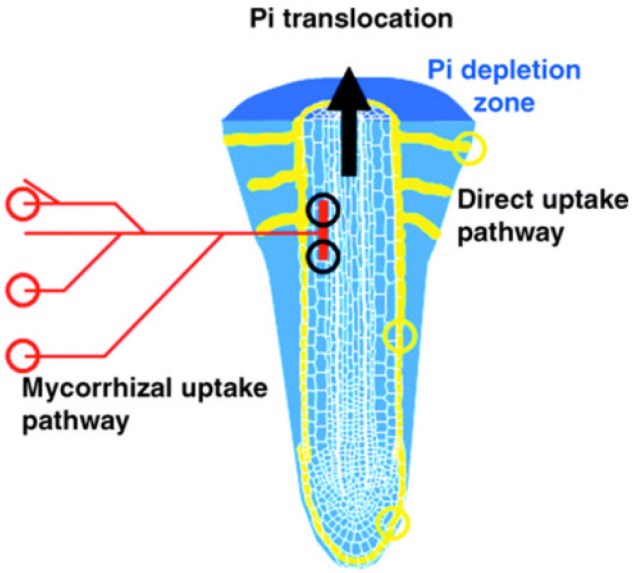

Figure 3.2-1 Two phosphate uptake pathways of mycorrhizal plants(adopted from Bucher, 2006). In direct uptake pathway, phosphate is absorbed via the root epidermis and root hairs (yellow circles). The direct uptake rate usually exceeds the diffusion rate and resulted in a phosphate depletion zone (blue zone) surrounding the root surface. In mycorrhizal uptake pathway, phosphate is taken up by mycorrhizal fungal hyphae (red circle) that can bridge the depletion zone and then translocated to the interface in the root cortex (black circle).

Based on these findings, it was hypothesized that the impact of a mutation in *MtHA1*on the symbiosis will be modulated by phosphate concentrations in the fertilizer and by the AM fungal species used for mycorrhization. To test this hypothesis, the homozygotes mutant (*MtHA1exon8::Tnt1*) and the corresponding wild type were grown under three different phosphate fertilizer concentrations and inoculated with the two AM fungi *R. irregularis* and *F. mosseae*. Four weeks after inoculation, plant growth and mycorrhization parameters, as well as phosphate content and gene expression pattern were compared between the mutant and the wild type.

3.2.2 Results

3.2.2.1 Plant growth

Germinated mutant and wild type seedlings were inoculated with *F. mosseae* or *R. irregularis* when transplanting to growth substrate and grown in parallel with mock-inoculated control plants at different phosphate fertilizer levels (1%, 10%, and 100% KH_2PO_4 of the usual fertilizer concentration). Plant growth parameters were measured four weeks after inoculation (**Table 3.2-1**). When plants were growing under low phosphate conditions (fertilized with 1% and 10% KH_2PO_4), both mycorrhizal fungi improved plant growth and, shoot/root ratio was higher in inoculated plants than in controls. At 1% phosphate, the mutation of *MtHA1* did not affect the growth promoting effect as MGD in mutant and wild type plants were not different. With 10% of the usual phosphate concentration in the fertilizer, the mutant plants showed a slight, but significant higher dependency on mycorrhization with *F. mosseae* showing higher MRI and MGD values than *R. irregularis*. When wild type plants were grown under optimal phosphate fertilizer conditions (100%), MRI and MGD for *F. mosseae* disappeared, while there was still a MGD of 34% for *R. irregularis*. MRI was not affected in the mutant, but both fungi showed MGD. Three-way ANOVA indicated that phosphate concentration in the fertilizer had a significant influence on whole plant growth while AM fungi only affected shoot biomasses. The mycorrhiza increased shoot/root ratio was affected by both phosphate concentration in the fertilizer and plant genotype. The *M. truncatula* growth dependency on AM fungi showed no difference between *F. mosseae* and *R. irregularis.*

3.2.2.2 Mycorrhiza formation

Mycorrhiza parameters of *M. truncatula* seedlings that fertilized with different phosphate concentration (1%, 10% and 100%) were determined four weeks after inoculation (**Table 3.2-2**). All mock-inoculated plants showed no fungal structures at all (data not shown). When fertilized with 1% and 10% phosphate, all mycorrhiza parameters of wild type plants were higher than those of mutant plants, but *F. mosseae* colonization was more affected by the *MtHA1* mutation than *R. irregularis* colonization and this was significant for F% (interaction between the factors plants genotype and fungal species). All differences were significant except the values of a% for *F. mosseae* or of F% for *R. irregularis* at 10%. When fertilized with 100% phosphate, all mycorrhiza parameters were lower, but differences between wild type and mutant plants were only significant in case of *F.mosseae* for m% and in case of *R. irregularis*-inoculated plants for M%, m% and A%. Generally, mycorrhizal parameters of *R.*

irregularis were higher than that of *F. mosseae* and statistical analysis indicated that this was significant for F%, M% and m%. Furthermore, phosphate concentrations in the fertilizer had significant impact on F%, M% and A%, while the genotype of *M. truncatula* significantly affected all mycorrhization parameters. Differences between wild type and mutant plants were lower at higher phosphate concentrations in the fertilizer and a significant interaction between the two factors was detected for a%.

Table 3.2-1 Influence of *F. mosseae* and *R. irregularis* at three phosphate fertilizer levels on growth parameters of wild type and *MtHA1 exon8::Tnt1* mutant plants. Average values of different parameters ± standard deviation are shown.Significant influences of inoculum type (myc), genotype and phosphate level (P_i) and significant interactions between the three factors are indicated. FW, fresh weight; DW, dry weight; MRI, mycorrhiza shoot/root ratio increase; MGD, mycorrhiza growth dependency; WT, *M. truncatula* wild type; MUT,*M. truncatula MtHA1 exon8::Tnt1* mutant; NM, without inoculation; FM, inoculated with *F. mosseae*; RI, inoculated with*R. irregularis*.

P_i fertilization	treatment	shoot FW (g)	root FW (g)	shoot DW (g)	shoot/root	MRI(%)	MGD (%)
1% P_i	WT NM	0.92±0.08	1.8±0.04	0.14±0.01	0.51±0.04	-	-
	WT FM	1.3±0.31	1.74±0.19	0.19±0.04	0.74±0.13	1.46±0.24 cd	24.07±15.29
	WT RI	1.22±0.26	1.79±0.38	0.22±0.05	0.69±0.07	1.36±0.14 d	31.02±14.89
	MUT NM	1.03±0.22	2.14±0.49	0.14±0.04	0.48±0.03	-	-
	MUT FM	1.28±0.43	1.74±0.46	0.22±0.07	0.72±0.07	1.50±0.15 bcd	28.78±24.6
	MUT RI	1.16±0.24	1.53±0.4	0.21±0.05	0.77±0.09	1.60±0.19 abc	28.9±19.16
10% P_i	WT NM	0.85±0.19	1.71±0.36	0.13±0.03	0.5±0.04	-	-
	WT FM	1.38±0.21	1.88±0.38	0.21±0.03	0.74±0.14	1.50±0.28 bcd	38.63±8.69
	WT RI	1.19±0.11	1.51±0.19	0.2±0.01	0.8±0.15	1.62±0.31 ab	36.15±4.04
	MUT NM	0.85±0.26	1.77±0.74	0.13±0.05	0.51±0.1	-	-
	MUT FM	1.59±0.06	1.91±0.23	0.29±0.01	0.84±0.08	1.70±0.30 a	53.79±2.08
	MUT RI	1.36±0.1	1.84±0.17	0.24±0.01	0.74±0.06	1.50±0.25 bcd	45.79±2.69
100% P_i	WT NM	1.47±0.19	2.17±0.74	0.23±0.02	0.71±0.13	-	-
	WT FM	1.68±0.31	2.37±0.19	0.26±0.05	0.71±0.1	1.03±0.22 f	11.4±14.4
	WT RI	2.02±0.24	2.69±0.31	0.35±0.04	0.75±0.05	1.09±0.20 ef	33.83±7.3
	MUT NM	1.48±0.2	2.38±0.44	0.21±0.02	0.63±0.05	-	-
	MUT FM	2.21±0.11	3.02±0.23	0.37±0.02	0.74±0.07	1.18±0.13 e	42.44±3.79
	MUT RI	2.03±0.28	2.79±0.11	0.36±0.05	0.73±0.08	1.17±0.14 ef	40.01±7.75
P_i		s	s	s	ns	s	s
genotype		ns	ns	s	ns	s	s
myc		s	ns	s	s	*ns	*ns
P_i*genotype		ns	ns	ns	ns	ns	ns
P_i*myc		ns	s	s	s	ns	ns
genotype*myc		ns	ns	s	ns	ns	ns
P_i*genotype*myc		ns	ns	ns	ns	s	ns

Note: s, significant influence or interactionat $P = 0.05$, $n = 4$; ns, not significant at $P = 0.05$, $n = 4$, *ns, not significant between two AM fungi

In addition to mycorrhization parameters, arbuscule morphology was also monitored (**Figure 3.2-2**). As already observed at six weeks (**Figure 3.1-2**), arbuscules in wild type plants were fully branched while in mutant plants were small and branches were rarely observed. These differences of arbuscule morphology between wild type and mutant were independent of phosphate concentration in the fertilizer and of the AM fungal species.

Table 3.2-2 Mycorrhization parameters of wild type and *MtHA1exon::Tnt1* mutant plants with *F. mosseae*or*R. irregularis* under different phosphate fertilization. Average values of different parameters ± standard deviation are shown.Three-way ANOVA indicated no significant interaction of all three factors, but plant genotype interacted with AM fungal species for the parameter F% and with the phosphate level in the fertilizer for a%. WT, *M. truncatula* wild type; MUT,*M. truncatula MtHA1 exon8::Tnt1* mutant; FM, inoculated with *F. mosseae*; RI, inoculated with *R. irregularis*; F%, infection frequency; M%, absolute mycorrhization intensity; m%, relative mycorrhization intensity; a%, relative arbuscule abundance; A%, absolute arbuscule abundance.

P_i fertilization	treatment	F%	M%	m%	a%	A%
1% P_i	WT FM	85.57±17.12	45.22±18.44	50.8±14	40.62±12.68	20.02±10.94
	WT RI	90.85±10.62	45.72±23.96	48.67±20.93	35.72±9.96	14.83±5.01
	MUT FM	39.75±8.16	6.58±2.76	16.82±7.13	7.79±2.02	0.54±0.27
	MUT RI	68.44±9.04	21.43±8.25	30.54±8.07	20.69±7.59	4.87±3.25
10% P_i	WT FM	79.68±16.09	36.71±15.23	44.53±11.03	24.91±9.52	9.51±4.76
	WT RI	91.23±11.09	54.2±31.38	56.99±28.09	37.74±12.32	20.99±14.29
	MUT FM	42.15±5.7	9.44±4.35	21.89±7.19	15.02±5.14	1.58±1.3
	MUT RI	85.56±6.01	31.17±4.5	36.39±3.95	19.72±3.99	6.03±0.66
100% P_i	WT FM	59.39±28.57	21.21±13.79	34.32±9.05	23.09±10.96	5.23±5.57
	WT RI	79.32±13.34	32.54±19.34	38.76±18.32	24.34±6.86	8.54±5.8
	MUT FM	42.59±13.57	7.78±3.67	17.98±4	18.31±9.72	1.44±0.92
	MUT RI	61.74±5.27	15.3±6.13	24.4±7.77	22.3±7.48	3.26±1.09
P_i		s	s	ns	ns	s
geno		s	s	s	s	s
myc		s	s	s	ns	ns
P_i*geno		ns	ns	ns	s	ns
P_i*myc		ns	ns	ns	ns	ns
geno*myc		s	ns	ns	ns	ns
P_i*geno*myc		ns	ns	ns	ns	ns

Note: s, significant influence or interactionat $P = 0.05$, $n = 4$; ns, not significant at $P = 0.05$, $n = 4$.

3.2.2.3 Phosphorus uptake

Phosphorus (P) concentrations were measured in shoots of *F. mosseae* or *R. irregularis* inoculated wild type and mutant plants and in the corresponding controls cultivated four weeks at different phosphate fertilizer levels. Based on the data obtained and on the dry weight of shoots (**Table 3.2-1**), P uptake was calculated (**Figure 3.2-3**). When plants were

fertilized with low amounts of phosphate (1% and 10% phosphate), inoculation of AM fungi increased uptake by increasing shootbiomass but not phosphorus concentration.When fertilized with 100% phosphate, P concentration and uptake were generally higher and P concentrations were reduced by both AM fungi if compared to non-inoculated control plants. This decrease was compensated by a biomass incensement resulting in similar values for P uptake. No differences were observed at this time point between wild type and mutant plants.

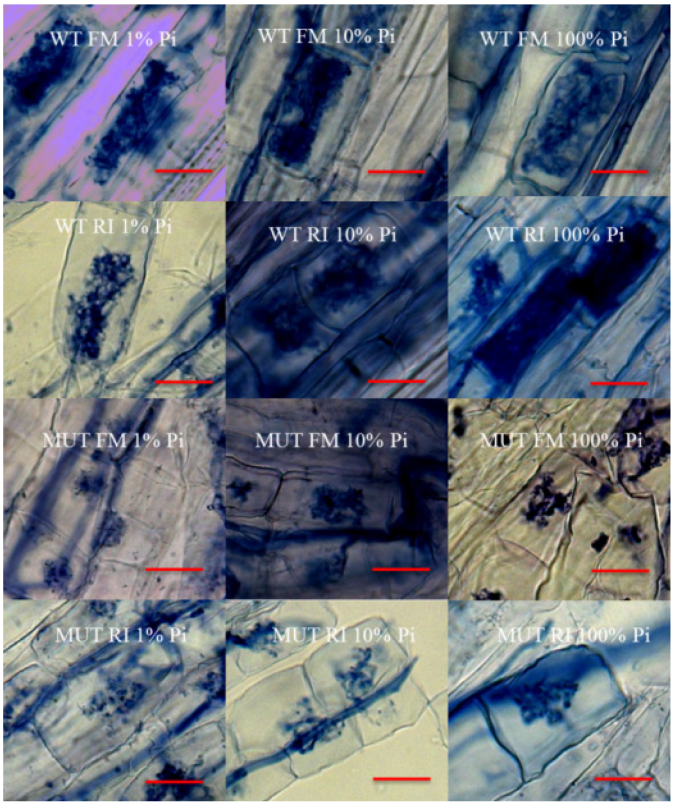

Figure 3.2-2 Arbuscules formed in *M. truncatula* roots of wild type and *MtHA1exon8::Tnt1* mutant plants. Four weeks after inoculation, arbuscules in wild type plants were fully branched, but in mutant plants, the arbuscules were small and the branches were rare. Phosphate fertilization levels and AM fungal species did not influence arbuscule morphology. WT, *M. truncatula* wild type; MUT,*M. truncatula MtHA1 exon8::Tnt1* mutant; FM, inoculated with *F. mosseae*; RI, inoculated with *R. irregularis.* Bars = 20 µm.

3.2.2.4 Relative gene expression

For functional analysis of the cells surrounding *F. mosseae* and *R. irregularis* arbuscules in wild type and mutant plants at different phosphate fertilization levels, the expression of three *M. truncatula* genes were studied by quantitative real-time PCR (**Figure 3.2-4**). Under low phosphate fertilization (1% and 10%), the mycorrhiza-specific induced phosphate transporter gene *MtPT4* was highly induced in wild type plants and this induction was strongly reduced when plants were fertilized with 100% phosphate. This pattern was independent of AM fungal

species. In the mutant plants, the *MtPT4* gene was only very slightly induced and this induction was higher in case of *R. irregularis*. Based on arbuscule number, suppression of relative *MtPT4* expression at higher phosphate levels and in mutant plants was still detectable, but not so obvious anymore.

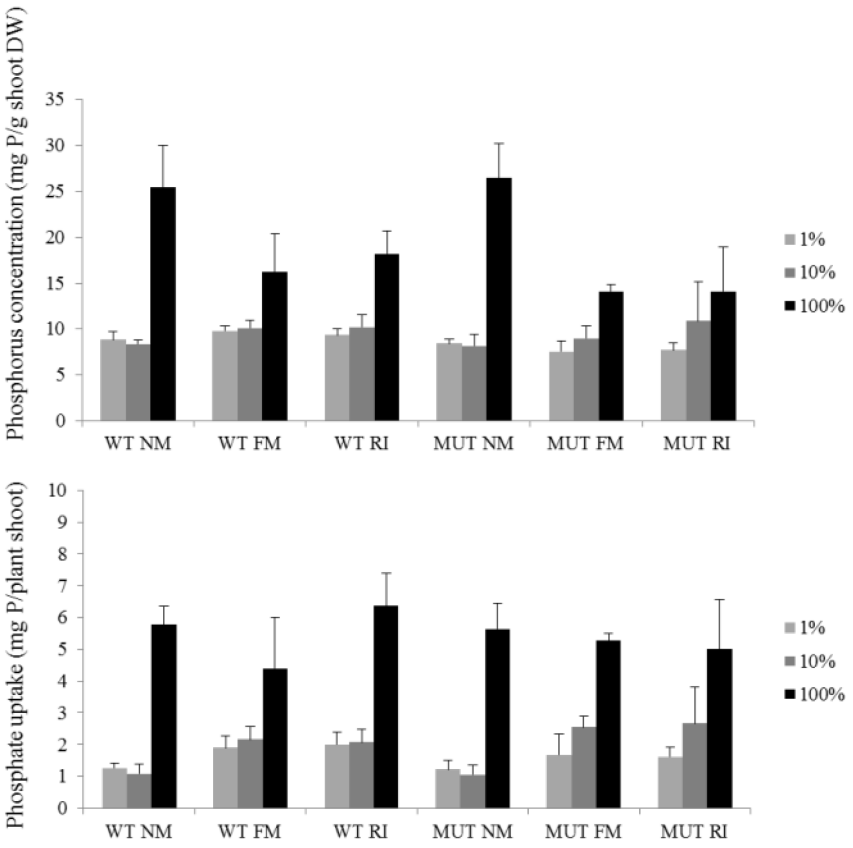

Figure 3.2-3 Influence of mycorrhization with *F. mosseae* and *R. irregularis* on shoot phosphorus (P) concentrations and uptake at different phosphate fertilization levels four weeks after inoculation. Uptake were calculated in *M. truncatula* mutant and wild type plants based on measurements of phosphorus concentrations and shoot dry weights (P uptake = P concentration x shoot dry weight). Shown are mean values and standard deviations. Analysis of variance indicated no significant interactions between the factors AM fungal species, genotype and phosphate fertilization levels, but both factors had significant influences on shoot P concentration and P uptake ($P = 0.05$; $n = 4$).WT, *M. truncatula* wild type; MUT,*M. truncatula MtHA1 exon8::Tnt1* mutant; NM, without inoculation; FM, inoculated with *F. mosseae*; RI, inoculated with *R. irregularis*, DW, dry weight.

The expression of *MtHA1* was significantly induced by *F. mosseae* at all phosphate fertilization levels in wild type plants, but in mutant plants this induction was marginal.*R. irregularis* showed a similar pattern, except that the induction in the mutant plants was higher than for *F. mosseae*. If related to arbuscule number, no significant differences were observed except that *MtHA1* expression was higher in arbuscules formed in mutants than those of wild type plants at lowest phosphate fertilization level (1%).

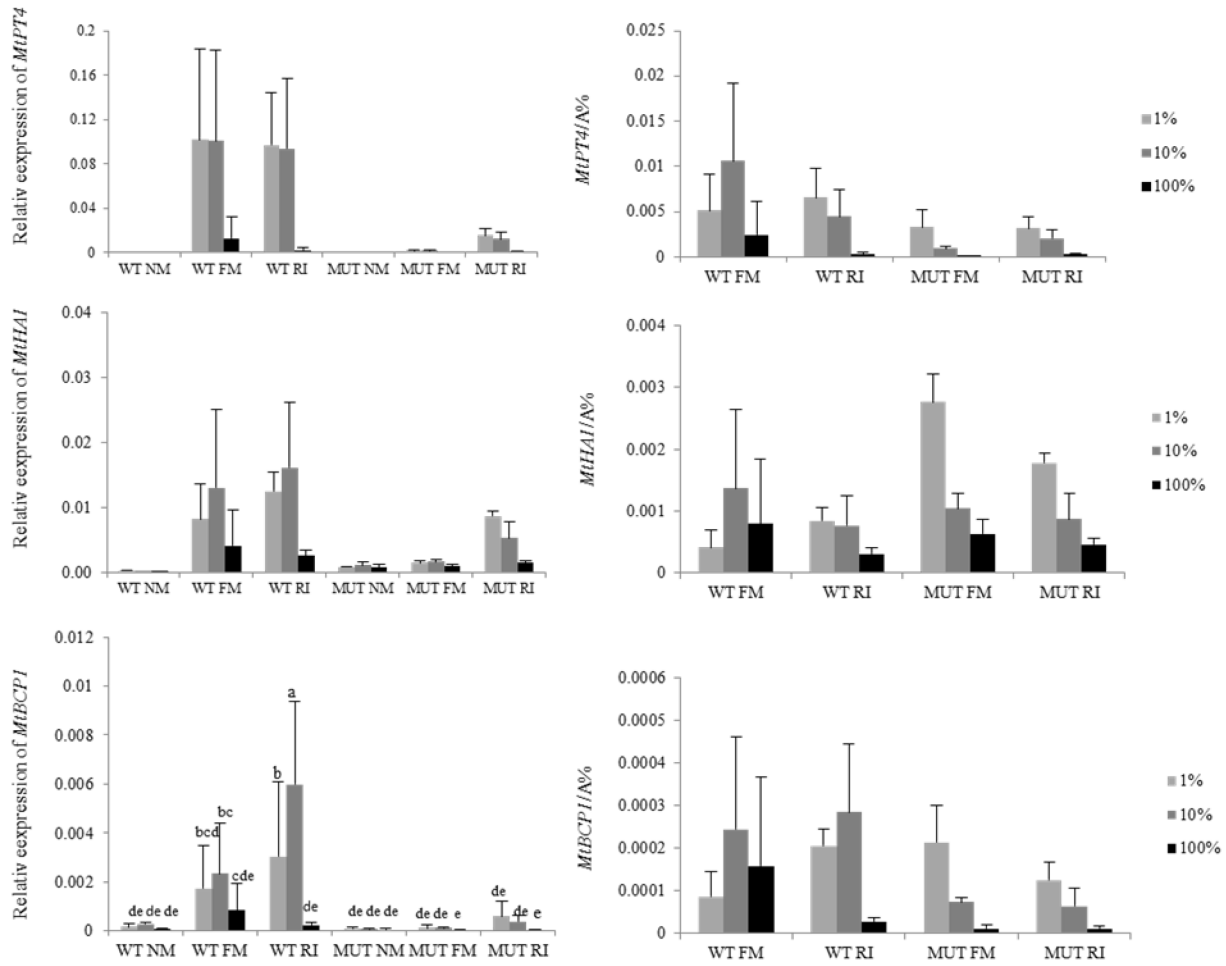

Figure 3.2-4 Gene expression levels of *MtPT4*, *MtHA1* and *MtBCP1*four weeks after inoculation. RNA was extracted from roots of wild type and *MtHA1 exon8::Tnt1* mutant plants inoculatedor not with *F. mosseae*or*R. irregularis*at different phosphate fertilization levels. Values obtained for the three arbuscule-specific genes were normalized with the values of *MtTEF1α* as reference gene (left graphs). Three-way ANOVA (*P* = 0.05, *n=3*) indicated that the factors Pi fertilization level, genotype and AM fungal species had significant effect on gene expression while significant interaction between factors was only observed for the expression of *MtBCP1*. Letters above columns indicate significant differences between treatments (LSD-test, *P* = 0.05, *n = 3)*. Right graphs show the ratio of RNA accumulation values for the three genes to arbuscule numbers observed in wild type and mutant plants. Three-way ANOVA (*P* = 0.05, *n = 3*) revealed no significant interaction between the factors (Pi fertilization level, genotype and AM fungal species) while Pi fertilization level and plant genotype had significant effect on the ratio.WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; NM, without inoculation; FM, inoculated with *F. mosseae*; RI, inoculated with *R. irregularis*. Bars = standard deviations.

The expression of the mycorrhiza-specific induced blue copper-binding gene *MtBCP1* was only induced by mycorrhization in wild type plants at 1% and 10% phosphate fertilizer levels.

When arbuscule numbers were taken as basis, significant reduction in expression levels in mutant compared to wild type plants was only observed at 10% phosphate. At the other fertilization levels, differences were not significant.

3.2.3 Discussion

Phosphorus is a macro element for plants. Besides direct absorption of phosphate via root epidermis and root hairs, a mycorrhizal pathway exists in plants where phosphate is delivered via the symbiosis with AM fungi. In many studies, where plants were grown under low phosphate conditions, AM fungi generally improve plant biomasses mainly mirrored by shoot growth promotion (Smith et al., 2011). In the current study, when *MtHA1* wild type and mutant plants were grown under low phosphate conditions (1% and 10% phosphate), the AM fungi *F. mosseae* and *R. irregularis* improved phosphate uptake and plant growth as shoot/root ratios increased in inoculated plants compared to the non-inoculated treatments. Mutation of *MtHA1* resulted in reduced mycorrhizal development and the suppressed arbuscule-related genes expression. When phosphate concentration in fertilizer reached the optimal level (100% phosphate), MRIwas abolished and mycorrhization parameterswere decreased to the lowest values. This is perhaps due to increased root growth and enhanced phosphate uptake via direct root absorption (Javot et al., 2007a). Phosphate uptake was improved under low phosphate conditions by AM fungal inoculation. When plants were grown under optimal phosphate conditions, the AM fungal-inoculated treatments did not show higher phosphate uptake than the non-inoculated treatments. This is in accordance with the results of Smith et al. (2004) who described that the phosphate uptake via mycorrhizal fungi is hidden under high phosphate fertilizer levels, but still functional. The abolished arbuscule-related gene expression indicated that in the current study the hidden phosphate uptake did not exist. Under low phosphate conditions, *MtHA1* mutation inhibited AM fungal development and arbuscule-related gene expression. High phosphate concentration in fertilizer had a similar effect as *MtHA1* in inhibiting AM fungal development and the expression of the three genes.

F. mosseae and *R. irregularis* were previously classified in the same genus *Glomus,* but recently separated by Schüßler and Walker (2010). The colonization of *R. irregularis* is usually faster than the other AM fungi (Alkan et al., 2006; Smith et al., 2004). In this study, regardless phosphate concentration and genotype of *M. truncatula*, the mycorrhization parameters of *R. irregularis* were higher than that of *F. mosseae*. Although different reactions

of host plants to *F. mosseae* and *R. irregularis* under different situations were reported (Ruiz-Lozano et al., 1995; Pozo et al., 1999; Christophersen et al., 2012), the promoted plant growth and phosphate uptake in this study showed no general difference between *F. mosseae* and *R. irregularis* inoculated treatments. In the inoculated wild type plants, the RNA accumulation of *MtPT4* and *MtHA1* did not differ between two AM fungi whiles the RNA accumulation of *MtBCP1,* which is located on the arbuscular trunk,was higher in the*R. irregularis* inoculated plants (Pumplin and Harrison, 2009). The similarity of the RNA accumulation and the mycorrhizal parameter evaluation under microscope further confirmed the character of *MtBCP1* as a molecular marker of arbuscule trunks (Parádi et al., 2010). Based on the first experiment (result **3.1**), it was assumed that in *MtHA1exon8::Tnt1* mutant plants, the development of arbuscules can start but did not succeed to form fine branches due to the lack of functional*MtHA1*. Thisrepresses the expression of genes encoding proteins localized in the periarbuscular membrane around arbuscule branches and this is regardless of the identity of the colonizing fungus.

The hypothesis that phosphate levels in the fertilizer influences the impact of the mutation in *MtHA1* could be confirmed. At higher phosphate levels, mycorrhization parameters including mycorrhiza-induced gene expression decreased in the wild type much more than in the mutant. The effects of the mutation in *MtHA1* were therefore much less pronounced at optimal phosphate fertilization level. Also the second hypothesis could be confirmed. Mycorrhization and mycorrhiza-regulated gene expression with both AM fungal species was reduced in the mutant compared to the wild type, but the symbiosis with *R. irregularis* was less affected than the symbiosis with *F. mosseae*.

3.3 Effect of*Tnt1*insertion in *MtHA1*exon8 on the formation of mycorrhiza with *Gigaspora rosea*, a fungus with strong carbon sink activity

3.3.1 Introduction

Arbuscular mycorrhizal fungi are obligate biotrophic microorganisms. They need host plants to complete their life cycle (Smith and Read, 2008). Under suitable conditions, the propagules (spores, separated intraradical or extraradical hyphae) can grow for a limited time. During this time, the main carbon storage compound (triacylglycerides and glycogen) of the fungus will be used to develop the coenocytic germ tubes and provide carbon skeletons for anabolism (Bago et al., 2000). If AM fungi do not meet host plants during this time, growth will stop accompanied with germ-tube septation and nuclear autolysis (Bago et al., 1998). If AM fungi meet an appropriate host plant, they will form a hyphopodium upon physical contact from which they enter plant roots and form typical mycorrhizal structures like arbuscules (Smith and Read, 2008). Two morphological types of mycorrhization can be observed: In the *Arum*-type, hyphae grow along cortex cells, and these intercellular hyphae penetrate cell walls and develop a highly branched arbuscule inside the apoplast of root cortex cells. In *Paris*-type, the intercellular phase is absent. Hyphae grow from cell to cell and form large coils with small intercalated arbuscules (Smith and Smith, 1997). From literature survey, the *Paris*-type mycorrhization occurs in more plant families than the *Arum*-type.The type of mycorrhization seems to be determined by the genomes of both partners and is not under environmental control(Smith and Smith, 1997; Carvagnaro et al., 2001). In early confocal microscopy observation, the coils that formed in root cells by *Paris*-types mycorrhization had a great surface area as the arbuscules formed by *Arum*-type mycorrhization (Dickson and Kolesik, 1999). As the coils were formed in consecutive cells, the fungal biomass of *Paris*-type mycorrhization per infected root length was much higher than the *Arum*-type in which the arbuscules were much scattered (Cavagnaro et al., 2003).

While AM fungi supply host plant mineral nutrients especially phosphorus, they receive most or all of their carbon from their hosts (Bago et al., 2003). Up to 20% of host plant photoassimilates can be transferred towards AM fungi (Ferrol et al., 2002). Root cells containing arbuscules act as a sink for sucrose, which is the main form of transported photoassimilates. This view is supported by *in situ* hybridization studies of genes encoding for enzymes involved in sucrose hydrolysis (Blee and Anderson, 1998). Hexose that hydrolyzed from sucrose can be taken up by AM fungi and finally transformed to storage compounds as

trehalose, glycogen and lipids (Shachar-Hill et al., 1995; Pfeffer et al., 1999).Different AM fungi species show different carbon sink effects. Species belonging to the genus *Gigaspora* usually exhibit stronger carbon sink effects than those of other genera. In split root studies with barley and sugar maple, *G. rosea* showed strong carbon sink effect while *F. mosseae* did not show any effect on carbon partitioning. The strong carbon sink effectof *G. rosea* is discussed to be due to its rapidly formed dense and extensive mycelium by its *Paris*-type mycorrhization (Lerat et al., 2003).

Based on the limited phosphate uptake in mycorrhizal *MtHA1* mutant (result **3.1** and **3.2**), on the model of mutual controlled nutrient exchange (Kier et al., 2010), and of the assumed large carbon demand of *G. rosea*,it was hypothesized that the *Paris*-type mycorrhization formed by *G. rosea* will be more severely reduced by *MtHA1* mutation.In order to test this hypothesis, the homozygous mutant (*MtHA1exon8::Tnt1*) and the corresponding wild type were inoculated with *G. rosea* and grown under low phosphate condition. Plant growth, mycorrhization and phosphorus uptake were then compared between the mutant and wild type plants.

3.3.2 Results

3.3.2.1 Plant growth

Mutant and wild type seedlings were inoculated with *G. rosea* and grown together with mock-inoculated control plants under conditions as mentioned above. Plant growth parameters were measured four weeks after inoculation (**Table 3.3-1**). In this experiment, growth parameters of wild type plants reached higher values than those of the mutant plants as the genotype had a significant effect on shoot fresh weight, root fresh weight and shoot dry weight. *G. rosea* possessed a slight negative impact on plant growth and shoot/root ratio, but this effect was not significant. This negative impact resulted in values below one for MRI and negative values for MGD. The negative MGD value seemed to be more pronounced in wild type plants, but due to high standard deviations, the difference was not significant.

3.3.2.2 Mycorrhizas formation

Four weeks after inoculation, mycorrhizal parameters of *M. truncatula* seedling were assessed in stained root samples (**Table 3.3-2**). No mycorrhizal structures were observed in the mock-inoculated plants (data not shown). F%, M%, a% and A% showed no difference between

inoculated wild type and mutant plants, but m% of wild type plants was significantly higher than that of mutant plants.

In *G. rosea* inoculated wild type plants, fine branches were observed in the root cells that harbor typical *Paris*-type hyphal coils. In mutant plants, however, only the hyphal coils could be detected (**Figure 3.3-1**)

Table 3.3-1 Impact of *G. rosea*on growth parameters of wild type and *MtHA1 exon8::Tnt1* mutant plants. Average values of different parameters ± standard deviation are shown.Two-way ANOVA revealed that plant genotype, but not mycorrhization had a significant influence on plant growth, but the interaction of genotype and mycorrhization was not significant for all parameters.FW, fresh weight; DW, dry weight; MRI, mycorrhiza shoot/root ratio improvement; MGD, mycorrhiza growth dependency; WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; G, inoculated with *G. rosea*; NM, without inoculation.

treatment	shoot FW (g)	root FW (g)	shoot DW (g)	shoot/root	MRI	MGD (%)
WT NM	0.76±0.05	0.81±0.07	0.1±0.01	0.95±0.08	-	-
WT G	0.68±0.15	0.76±0.21	0.09±0.02	0.91±0.19	0.96±0.19	-16.83±36
MUT NM	0.61±0.1	0.59±0.11	0.08±0.01	1.03±0.1	-	-
MUT G	0.51±0.08	0.58±0.12	0.08±0.01	0.9±0.07	0.87±0.10	-9.09±11.56
genotype	s	s	s	ns	ns	ns
myc	ns	ns	ns	ns	na	na
genotype*myc	ns	ns	ns	ns	na	na

Note: s. significant difference or interaction at $P = 0.05$, $n = 4$; ns, no significant difference at $P = 0.05$ $n = 4$; na, not applicable.

Table 3.3-2 Mycorrhization parameters of wild type and *MtHA1 exon8::Tnt1* mutant plants with *G. rosea*. Average values of different parameters ± standard deviation are shown. In mock-inoculated samples, no mycorrhizal structures were observed. One-way ANOVA indicated significant effect of plant genotype on relative mycorrhization intensity WT,*M. truncatula* wild type; MUT,*M. truncatula MtHA1 exon8::Tnt1* mutant; NG, without inoculation; G, inoculated with *G. rosea*; F%, infection frequency; M%, absolute mycorrhization intensity; m%, relative mycorrhization intensity; a%, relative arbuscule abundance; A%, absolute arbuscule abundance.

treatment	F%	M%	m%	a%	A%
WT G	59.07±16.26	11.48±5.42	18.08±5.11	37.8±9.57	4.48±2.48
MUT G	59.72±6.05	5.69±1.54	9.4±2.01	33.16±11.92	1.89±0.95
genotype	ns	ns	s	ns	ns

Note: s. significant difference at $P = 0.05$, $n = 4$; ns, no significant difference at $P = 0.05$ $n = 4$.

3.3.2.3 Phosphorus uptake

Phosphorus (P) concentrations were measured in shoots of *G. rosea*-inoculated plants and in corresponding controls after four weeks. Based on the data obtained and on the dry weight of shoots (**Table 3.3-2**), phosphorus uptake was calculated (**Figure 3.3-2**). Regardless of genotype and mycorrhization, neither P concentration nor P uptake showed differences among treatments.

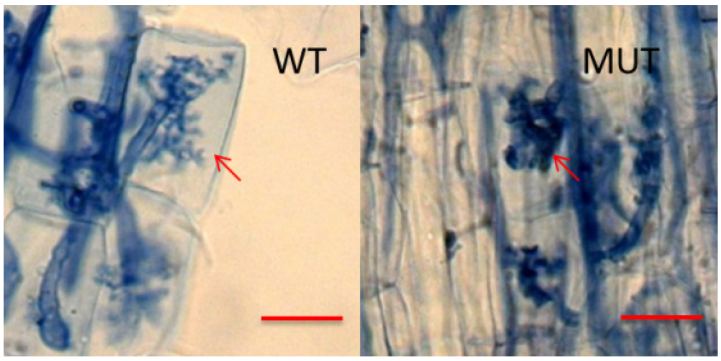

Figure 3.3-1 Arbuscules formed in *M. truncatula* root of wild type and *MtHA1exon8::Tnt1* mutant plants. Four weeks after inoculation of *G. rosea,* in wild type plants (left) branched arbuscules of *Paris*-type (red arrow), while in mutant plants (right), only coiled hyphae were observed (red arrow). WT,*M. truncatula* wild type; MUT,*M. truncatula MtHA1 exon8::Tnt1*mutant. Bars = 20 µm.

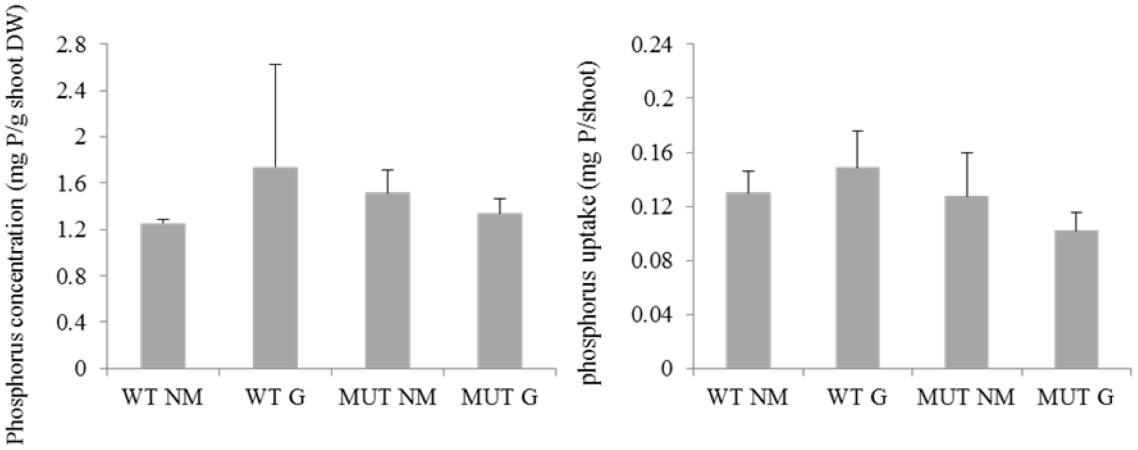

Figure 3.3-2 Influence of mycorrhization with *G. rosea*on shoot phosphorus(P) concentration and uptake. Uptake were calculated in *M. truncatula*MUT and WT plants based on measurements of phosphorus concentrations and shoot dry weights (P uptake= P concentration x shoot dry weight). Shown are mean values and standard deviations. Based on two-way ANOVA, genotype and mycorrhization had no significant effect on P concentration and P uptake in WT and MUT plants. WT,*M. truncatula* wild type; MUT,*M. truncatula MtHA1 exon8::Tnt1* mutant; G, inoculated with *G. rosea*; NM, without inoculation. Bars = standard deviations.

3.3.3 Discussion

As many experiments have shown, mycorrhization of plants by isolates belonging to the genus *Gigaspora* is different from the patterns which can be observed with isolates from other genera (e.g. former *Glomus*). At first, infection by AM fungi within the genus *Glomus* and host plants can start from propagules as from spores, extraradical hyphae and infected roots, while in case of *Gigaspora* only spores can serve for this purpose (Smith and Read. 2008). In the present experiment, four weeks after inoculation (only spores), mycorrhization parameters of *G. rosea* were generally smaller compared with *F. mosseae* and *R. irregularis* in previous experiments (result **3.1** and **3.2**) and this was especially true for the absolute arbuscules abundance. This is consistent with the general observation that the formation of mycorrhizas by *G. rosea* is relatively slow (Ezawa et al., 1995; Boddington and Dodd, 1999; Burleigh et al., 2002; Smith et al., 2003; Cavagnaro et al., 2005). Secondly, unlike the species within the genus*Glomus* which form *Arum*-type, *Paris*-typeor mixed types of mycorrhization in different host plant roots, *G. rosea* onlyforms *Paris*-type mycorrhization patterns (Cavagnaro et al., 2001; Dickson. 2004; Smith et al., 2004). In the present study, the combination of *M. truncatula* and *G. rosea* resulted in a clear *Paris*-type mycorrhization. Although the absolute arbuscules abundance showed no difference between inoculated wild type and *MtHA1exon::Tnt1* mutant plants, morphological differences were observed. In wild type plants, hyphal coils were arbusculated while only hyphal coils were formed in mutant plants. Using double staining, Dickson (2004) indicated that the arbusculated coils are structurally similar to arbuscules and probably play the same role as *Arum*-type arbuscules in nutrient transfer. The loss of arbusculation of coils can therefore be seen as a homologous reaction of the fungus to the loss of *MtHA1* function as the truncated arbuscules of *F. mosseae* and *R. irregularis* in result**3.1** and **3.2**.

Although the infection frequency of *G. rosea* was relatively lower than *F. mosseae* and *R. irregularis* in previous experiments, the *Paris*-type mycorrhization in which the coils were formed in consecutive cells might result in a larger fungal biomass inside roots (Cavagnaro et al., 2003). As most or all carbon of AM fungi was deprived from plants, the large amount of fungus would present a notable carbon sink for the host.Inoculation of *G. rosea*in this study slightlysuppressed plant growth which is similar to others studies including *M. truncatula* (Burleigh et al., 2002; Lerat et al., 2003).Besides intraradical growth, the characters of *G. rosea*to produce large spores and auxiliary cells by extraradical mycelium also suggest that this fungus needs large amounts of carbon andexplains that it suppresses plant growth

(Boddington and Dodd, 1999). The ability of AM fungi to supply phosphate to host plants is also different between genera (Smith et al., 2003). In the current study, inoculation of *G. rosea* did not improve phosphorus concentration and uptake and this confirms the result of Burleigh et al. (2002) who assessed the expression of plant P-starvation genes which are reduced in *Glomus*-, but not in *G. rosea*-inoculated plants. This could be explained by the limited ability of *G. rosea* to absorb phosphate at distant sites from the root as other species e.g. *R. irregularis* can do (Cavagnaro et al., 2005). Smith et al. (2003) reported that *G. rosea* contributes only low amounts of phosphate, but increased direct phosphate uptake via root epidermis and root hairs which is down-regulated by other AM fungi.

Within the arbuscular mycorrhiza, both plant and AM fungi cancontrol the amount of released nutrient that the partner needs (Kier et al., 2011). As a less cooperative AM fungus, *G. rosea* requires large amounts of carbon and supplies little amounts of phosphate to *M. truncatula* (Smith et al., 2003). In order to limit this behavior, plant will reduce the infection of *G. rosea* which is observed inthe current study and described in other reports (Ezawa et al., 1995; Boddington and Dodd, 1999; Burleigh et al., 2002; Cavagnaro et al., 2005). When plants reduce carbon supply, AM fungi will store nutrientslike phosphate in their extraradical hyphae and spores (Hammer et al., 2011). Under nutrient limited condition, the extraradical nutrient storage of AM fungi and its stronger capability of nutrient absorption than plant roots will further aggravate plant nutrient deficiency. In order to avoid this situation, plant will then control the infection of *G. rosea* to an extent in which the phosphate uptake is at the similar level with non-infected status by sacrificing part of its growth. This situation will change, however, after the fungus is fully developed. For instance, four weeks after inoculation *G. rosea* suppressed plant growth and the suppression disappeared after eight weeks growth (Lerat et al., 2003). Although the phosphate uptake via mycorrhizal pathway in the *MtHA1* mutant plants is limited (result **3.1** and **3.2**), *G. rosea* could still form some mycorrhization. One explanation could be the compromise made by plant to release the intense nutrient storage of extraradical hyphae. In another *M. truncatula* mutant, silencing the mycorrhizal specifically induced phosphate transporter (*MtPT4*) resulted in the pre-mature andsenescence of arbuscules and terminated symbiosis (Javot et al., 2007b). Combining these results, it is assumed that either there is still phosphate uptake via the mycorrhizal pathway or other nutrients such as nitrogen are supplied by *G. rosea* to support the symbiosis (Javot et al., 2011).

In summary, the hypothesis that*MtHA1* mutation affects mycorrhization with *G. rosea* more than the interaction with *F. mosseae* and *R. irregularis* has to be rejected. In contrast, the impact on the symbiosis seems to be less. The reason could be that the fungus does not provide already the wild type plants with much phosphate in the current situation. Therefore, the transfer of carbohydrates towards the fungus is also reduced compared to the other two fungi resulting in lower mycorrhization parameters. Any mutation affecting the mycorrhizal phosphate uptake and in consequence the carbohydrate supply of the fungus has not such a big effect as if the nutrient exchange between wild type plant and mycorrhizal fungus reaches high amounts.

3.4 Effect of the *Tnt1* insertion in *MtHA1 exon8* on nodule formation and function

3.4.1 Introductions

Medicago truncatula originated from the Mediterranean region was chosen as a model plant based on its small, diploid genome, on its self-fertility and its rapid and prolific seed production, and on its amenability to genetic transformation (Cook, 1999). Unlike the standard model *Arabidopsis thaliana*, this legume plant forms arbuscular mycorrhiza and the root nodule symbiosis (RNS) with rhizobia. Although RNS is restricted to leguminous plants unlike AM, which can be found in more than 80% of terrestrial plants, and although a complete new and obvious organ (nodule) is formed, which is absent in AM, these two symbioses share common and similar aspects (Oldroyd and Downiw, 2006; Parniske, 2008; Bapaume and Reinhardt, 2012).

Best investigated is the common signal transduction pathway at the early stages of both symbioses, the so-called SYM pathway. Before physical contact, diffusible signal molecules that are secreted by plant roots can be perceived by AM fungi (Aikyama et al., 2005) or by rhizobia (Firmin et al., 1986). In return, rhizobia produce specific symbiotic signals called NOD factors (NFs) (Hassan and Mathesius, 2012), while AM fungi secrete so-called MYC factors (MFs) (Maillet et al., 2011). NOD and MYC factors are in turn perceived by LysM-receptor-like kinases (SYMRKs), which were firstly identified in *M. sativa* and *L. japonicus* (Endre et al., 2002; Stracke et al., 2002). After the perception of NFs or MFs, calcium spiking triggers host cell transcriptional reprogramming (Oldroyd and Downiw, 2006). The calcium spiking induced by AM or RNS is decoded by a calcium-calmodulin-dependent protein kinase (CCaMK) (Oldroyd and Downiw, 2006), which is a component of the SYM pathway, and its substrate protein encoded by *CYCLOPS* is required for further progress of AM and RNS formation (Bapaume and Reinhardt, 2012).

When plants physically contact these two types of microorganisms (AM fungi and rhizobia), a series of similar root cell behaviors occur supporting the invasion of both symbionts. For RNS, a tubular hollow structure called infect thread is formed which guides the rhizobia to the inner cortical cells (Fournier et al., 2008; Van Brussel et al., 1992). For the AM symbiosis, a homologous structure called prepenetration apparatus (PPA), a thick cytoplasmic bridge across the vacuole of the host cell,is formed. Cytoskeletal microtubules, microfilaments and

dense endoplasmic reticulum cisternae form a hollow tube inside PPA that connects the leading nucleus with the site of the hyphpodial contact (Genre et al., 2005, 2008; Siciliano et al., 2007). This PPA whichis similar to the nodule infection thread allows the penetration of the root cell apoplast by the AM fungal hyphae.

Symbiosomes as the main component of endosymbioses are compartments within host cell where a plant-derived membrane partially or completely encloses the microorganisms in the AM symbiosis or in the RNS (Parniske, 2000). This plant-derived membrane is specialized for diverse functions in molecular communication and in nutrient exchange (Bapaume and Reinhardt, 2012). During formation of AM, the plant-derived periarbuscular membrane surrounds ramified fungal hyphae inside root cells forming the so called arbuscules (Smith and Read, 2008), which is the main site for the transfer of a range of mineral nutrients from AM fungi to host plants (Smith and Smith, 2011). In the process of RNS, bacteria will divide and differentiate to form the nitrogen-fixing bacteroids inside plant cells which are enclosed by the plant-derived peribacteroid membrane (Udvardi and Day, 1997).

H^+-ATPases that consume ATPs to form electrochemical gradients play an important role in nutrient transport across the plasma membrane (Palmgren, 2001). In AM, the transportation of phosphate across the periarbuscular membrane depends on a phosphate transporter and this process is driven by an electrochemical gradient provided by H^+-ATPase activity (Harrison et al., 2002; Kobae and Hata, 2010). Such an activity has been located on the membrane surrounding the arbuscules and was postulated to be essential for AM symbiotic functioning (Marx et al., 1982; Gianinazzi-Pearson et al., 1991). In RNS, NH_3 released from bacteroids by its concentration gradient is protonated to NH_4^+, which in turn can be transported across the peribacteroid membrane via an ammonium transporter (Udvardi and Day, 1997; Kaiser et al., 1998; Rogato et al., 2008). The protonation of NH_3 is a resultof H^+-ATPases located on the peribacteroid membrane (Udvardi and Day, 1997; Fedorova et al., 1999).

In previous studies, a gene (*MtHA1*) encoding H^+-ATPase was identified that specifically expressed in arbuscule containing cells of *M. truncatula* (Krajinski et al., 2002). The transposon insertion in exon 8 of this gene resulted in reduced phosphate uptake and abnormal arbuscules development (Result **3.1** and **3.2**) indicating its important function for the mycorrhizal symbiosis. Based on the similarity between AM and RNS, the important function of H^+-ATPase activity for both symbiosis, and the observation of the induced transcript levels

of *MtHA1* in root nodules (Manthey et al., 2004), it was hypothesized that *MtHA1* also affects the formation and function of nodules. To test this hypothesis, the homozygotes mutant (*MtHA1exon8::Tnt1*) and the corresponding wild type plants were grown under nitrogen free conditions and inoculated with nodule-forming rhizobia (*Sinorhizobium meliloti* in case of *M. truncatula*). Four weeks after inoculation, plant growth and nodule formation, as well as nitrogen content and gene expression pattern were compared between the mutant and the wild type plants.

3.4.2 Result

3.4.2.1 Plant growth and nodulation

Mutant and wild type seedlings were inoculated with *S. meliloti* and grown together with mock-inoculated control plants under conditions as mentioned above. Seedlings were fertilized with Hoaglands' solution containing no nitrogen. Plant growth parameters were measured four weeks after inoculation (**Table 3.4-1**). Inoculation of rhizobium improved plant growth as the inoculated plants had larger shoot and root biomasses and higher shoot/root ratios than the mock-inoculated plants. Rhizobium growth dependency (RGD) showed no difference between wild type and mutant plants. Mutation of *MtHA1* did not influence shoot growth, but increased root growth and hence decreased shoot/root ratios. No nodule was formed in mock-inoculated plants (data not shown). The number of nodules in wild type and mutant plant roots showed no significant difference. Analysis of variance indicated significant influence of rhizobium on plant growth parameters while genotype significantly affects root growth and shoot/root ratio. No significant interaction was observed between genotype and rhizobium for growth parameters.

3.4.2.2 Nitrogen uptake

Nitrogen concentrations were measured in shoots of rhizobium inoculated and mock-inoculated wild type and mutant plants at four weeks. Based on the data obtained and on the dry weight of shoots (**Table 3.4-1**), nitrogen uptake was calculated (**Figure 3.4-1**). Nitrogen concentration showed no significant difference among the four treatments while nitrogen uptake in rhizobium-inoculated plants was higher than in mock-inoculated plants. Two-way ANOVA indicated significant effects of rhizobium on nitrogen uptake, but the genotype did neither affect nitrogen concentration nor uptake.

Table 3.4-1 Impact of rhizobium inoculation and genotype on plant growth parameters and nodule formation. Average values of different parameters ± standard deviation are shown. Significant influence and interaction of inoculation with the nodule forming rhizobium *S. meliloti* and of the genotype is indicated. FW, fresh weight; DW, dry weight; RGD, rhizobium growth dependency; WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; R, inoculated with rhizobium; NR, without inoculation

Treatment	shoot FW (g)	root FW (g)	shoot DW (g)	shoot/root	nodule number	RGD (%)
WT NR	0.21±0.03	0.31±0.06	0.04±0.01	0.69±0.06	0	-
WT R	0.46±0.12	0.42±0.07	0.07±0.01	1.09±0.18	14±2	51.9±11.6
MUT NR	0.26±0.01	0.41±0.05	0.05±0.001	0.63±0.07	0	-
MUT R	0.48±0.14	0.57±0.12	0.09±0.03	0.84±0.12	19±10	43.0±16.8
genotype	ns	s	ns	s	ns	ns
rhizobium	s	s	s	s	na	na
genotype*rhizobium	ns	ns	ns	ns	na	na

Note: s, significant influence or interaction at $P = 0.05$, $n = 4$; ns, not significant at $P = 0.05$, $n = 4$; na, not applicable.

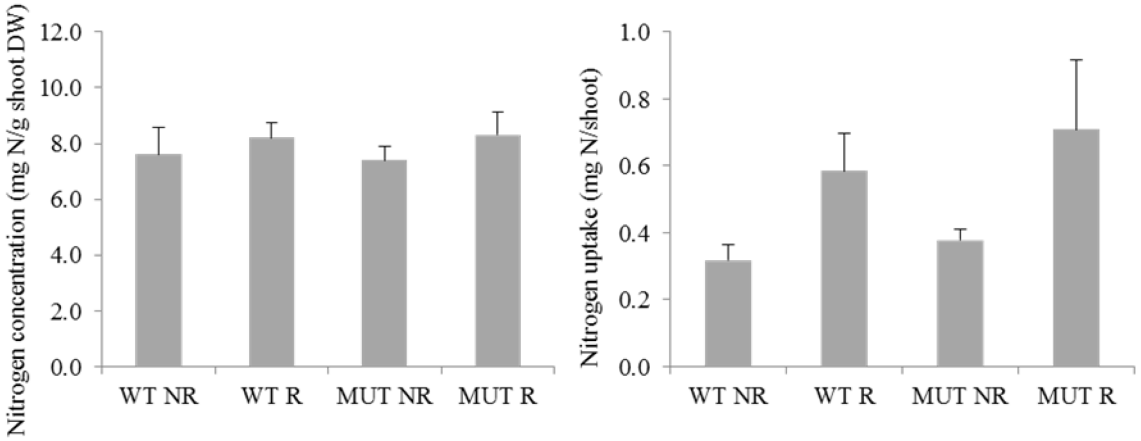

Figure 3.4-1 impact of rhizobium inoculation and genotype on nitrogen concentration and uptake. Uptake rate were calculated in *M. truncatula* mutant and wild type plants inoculated or not with the rhizobium *S. meliloti* based on measurement of nitrogen concentrations and shoot dry weight (Nitrogen uptake=nitrogen concentration × shoot dry weight). Shown are mean values and standard deviations. Two-way ANOVA showed significant effect of *S. meliloti* inoculation on nitrogen uptake, but no interaction was observed between the factors genotype and rhizobium. WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; R, inoculated with rhizobium; NM, without inoculation. Bars = standard deviations.

3.4.2.3 Relative expression of *MtHA1*

The relative expression of *MtHA1* was assayed four weeks after inoculation of plants with *S. meliloti*. There seemed to be a slight effect of inoculation (**Figure 3.4-2**), but differences were

not significant. Neither the genotype nor the inoculation considerably affected*MtHA1* RNA accumulation.

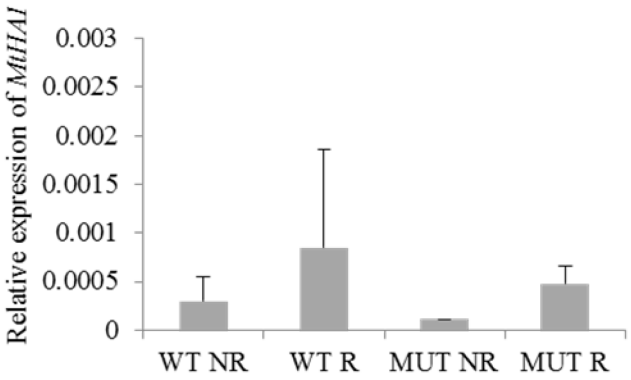

Figure 3.4-2 Gene expression level of *MtHA1*. RNA was extraction from roots (with nodules) of wild type and *MtHA1 exon8::Tnt1* mutant plants inoculated or not with *S. meliloti*. Values obtained for *MtHA1* gene were normalized with the value of *MtTEF1α* as the reference gene. Two-way ANOVA indicated no significant effect and no interaction of the factors genotype and rhizobium. WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; R, inoculated with rhizobium; NM, without inoculation. Bars = standard deviations.

3.4.3 Discussion

H^+-ATPases were found exclusively in the plasma membrane of fungi and plants (Duby and Boutry, 2009) and are in the latter located in the phloem, in stomatal guard cells, in the root epidermis and in root hairs, in pollen tubes, and in nodules and cells containing arbuscules (Marx et al., 1982; Gianinazzi-Pearson et al., 1991; Fedorova et al., 1999; Palmgren, 2001). They consume ATPs for pumping protons which leads to electrochemical gradients allowing active transport of solutes across membranes (Duby and Boutry, 2009). In plants, multigene families encode H^+-ATPases.For instance, twelve genes were detected in *Arabidopsis thaliana* (Palmgren, 2001). In *M. truncatula*, Southern blot analyses revealed a gene family with at least six members whereof three are expressed in roots, and one, *MtHA1*, was specifically expressed in arbuscule-containing cells (Krajinski et al., 2002).

Based on results of the previous studies (result **3.1** and **3.2**),the gene *MtHA1* is important for AM symbiotic formation and function. Nodule development is affected in many plant mutants that cannotform AM and vice versa (e.g. Gianinazzi-Pearson, 1996; Kosuta et al., 2003). *MtHA1* mutant plants were used to test the putative function of the gene in the nodule symbiosis. In this experiment, inoculation of *M. truncatula* wild type and mutant plants with *S.*

*meliloti*didnot significantly induce the expression of *MtHA1*. This was in contrast to a previousstudy (Manthey et al., 2004), where the *MtHA1* gene was even higher induced in nodules than in mycorrhizal roots. One possible explanation for the controversial results might be an RNA dilution effect, because RNA was extracted from pure nodules for the analyses carried out by Manthey et al. (2004) while roots with nodules were used for the present study.

According to Ivanov (2012), silencing *MtVAMP72s* genes encoding vesicle-associated membrane proteins resulted in rarely branched arbuscules similar to the phenotype observed in *MtHA1* mutant plants (result **3.1**, **3.2** and **3.3**). However, nodule formation and symbiosome development inside nodules is clearly affected in *MtVAMP72s*-silenced lines, while the number of nodules was not influenced by the *MtHA1* mutation. Accompanied with the rarely branched arbuscules, AM-promoted phosphate uptake was limited in *MtHA1* mutant plants (result **3.1** and **3.2**), but *MtHA1* mutation did not affect nitrogen uptake via nodules. Nodulation and nitrogen fixation is not affected in *MtHA1* mutant plants and this could indicate that the MtHA1 is either not the ATPase which has been located on the peribacteroid in nodules formed between *M. truncatula* and *S. meliloti* (Catalano et al., 2004) or it is not involved in the process of NH_3 protonation as described by Udvardi and Day (1997). Alternatively, ATPase activity provided by the *MtHA1*-encoded protein could be complemented in the mutant by other genes of the H^+-ATPase gene family. A number of such genes are expressed in nodules according to the *M. truncatula* Gene Expression Atlas (http://mtgea.noble.org/v2/) and the encoded proteins could be able to take over the function of MtHA1 in the symbiosis with the nitrogen-fixing bacteria. Such a complementation seems to be notpossible in case of the AM symbiosis. Based on these results, the hypothesis MtHA1 plays a role in the nodule symbiosis has to be rejected.

3.5 Analysis of the *Tnt1*insertion in exon7 of*MtHA1*

3.5.1 Introduction

Legumes are one of the largest crop families and contribute a large proportion of their nitrogen needs by their symbiotic interaction with nitrogen-fixing rhizobia (Graham and Vance, 2003). *M. truncatula* belongs to the subfamily Papilionoideae containing nearly all important legume crops, and is phylogenetically close to another model plant *L. japonicus* in the same clade Galegoides(Zhu et al., 2005). Small genome size and easy genetic transformation make *M. truncatula* a model plant in research on plant symbioses. For generating and analysis of *M. truncatula* mutants, severalapproaches like insertion mutagenesis, fast neutron bombardment, TILLING (targeting-induced local lesions in genomes), and RNA interference (RNAi) areapplicable (Trieu et al., 2000; d'Erfurth et al., 2003; Tadege et al., 2005, 2008; Stark et al., 2006; Le Signor et al., 2009; Limpens et al., 2004).

Differentmethodsare applied to induce mutations in plants. Unlike point chemical mutagens such as ethyl-methyl sulfonate (EMS) which inducesa broader range of mutant types including loss-of-function, gain-of-function, altered function, and novel function mutations, insertional mutations primarily produce loss-of-function mutants (d'Erfurth et al., 2003; Tadege et al., 2008). DNA-insertion mutagenesis using T-DNA, transposons or retrotransposonsopens the possibility to identifythe mutated gene of interest by using the inserted DNA sequence as "tag" (Pislariu et al., 2012). Application of T-DNA tagging, which was effectively used in Arabidopsis, was limited in legume mutagenesis as their large genome size need too much effort to achieve saturation (Tadege et al., 2008). The 'cut and paste' character of the class II DNA transposons (Ac/Ds, En/Spm and *MITE mPing*) makes the mutations in genomes unstable (Wessler, 2006; Hancock et al., 2011), and their intention of inserting at A/T-rich region that are often outside of coding sequences makes the mutant generation more difficult (Pislariu et al., 2012). Retrotransposons known as class I transposable elements that move in genomes via a 'copy and paste' mechanism can generate stable mutations during seed-to-seed propagation (Wessler, 2006; d'Erfurth et al., 2003). The long terminal repeat retrotransposon *Tnt1* from tobacco was used to generate insertion mutant over 19,000 lines in *M. truncatula*. In the first visible phenotype screening, approximated 30% visible mutants were identified in at least 25 distinct phenotypicclasses, including super-nodulation, non-nodulation, non-nitrogen-fixing, reduced mycorrhization, late and early

flowering, dwarf, altered anthocyanin content, light green, albino, transformed floral orangs and singlet leaves with cauliflower-like reproductive tissue (Tadege et al., 2008; http://medicago-mutant.noble.org/mutant/index.php).

In previous studies (result **3.1**, **3.2**, **3.3**, and **3.4**), the*MtHA1 exon8::Tnt1* homozygous mutant and the corresponding isogenic wild type were used to assess the function of the proton pumping ATPase gene (Krajinski et al., 2002). In addition to this mutant, a second *MtHA1* mutant allele was identified during the initial screen where the *Tnt1* transposon is inserted in exon 7. While exon8 is assumed to encode the amino acid region ofthe transmembrane domain M3 and M4, the corresponding exon7 encodes the peptide for the actuator domain (Palmgren et al., 2001). Comparison of*MtHA1 exon7::Tnt1* homozygous mutant with the corresponding wild type plants would be important to confirm the function of the *MtHA1* gene. In order to produce homozygous plants, seeds from heterozygous plants were germinated and cultivated in pots, and genotypes of seedlings wereidentified by conventional PCR.

3.5.2 Results

3.5.2.1 Selection of primer pairs for genotype identification

Based on sequencing of *Tnt1* flanking regions, the retrotransposon was presumably locatedin *MtHA1* exon7. Two primer pairs were used to check, if*M. truncatula* seedlings are homozygous wild type, homozygous mutated or heterozygous. Primer pair 1 (MtHA1-for and MtHA1-rev) was located on exon2 and exon10 (**Figure 3.5-1**), while primer pair 2 (Tnt1-for and Tnt1-rev) was located on the inverted repeat region of *Tnt1*. Two PCRs were needed to confirm the seedling genotype. First PCR was conducted with primer pair 1 and second PCR was conducted with one primer from primer pair 1 and one from primer pair 2. Based on the results of gel electrophoresis (**Figure 3.5-2**), the primer group 3 (MtHA1-for and Tnt1-rev) was chosen for the second PCR. Using DNA extracts from homozygous wild type plants will only result in an amplification product of 3308 ntin the first PCR (group 1 primers), while DNA from homozygous mutant plants will lead to a product of 2342 ntin the second PCR (group 3 primers). If amplification products can be obtained by both PCR reactions, seedlings are heterozygotes meaning that they carry the wild type allele and the allele with the*Tnt1* insertion in the *MtHA1* gene.

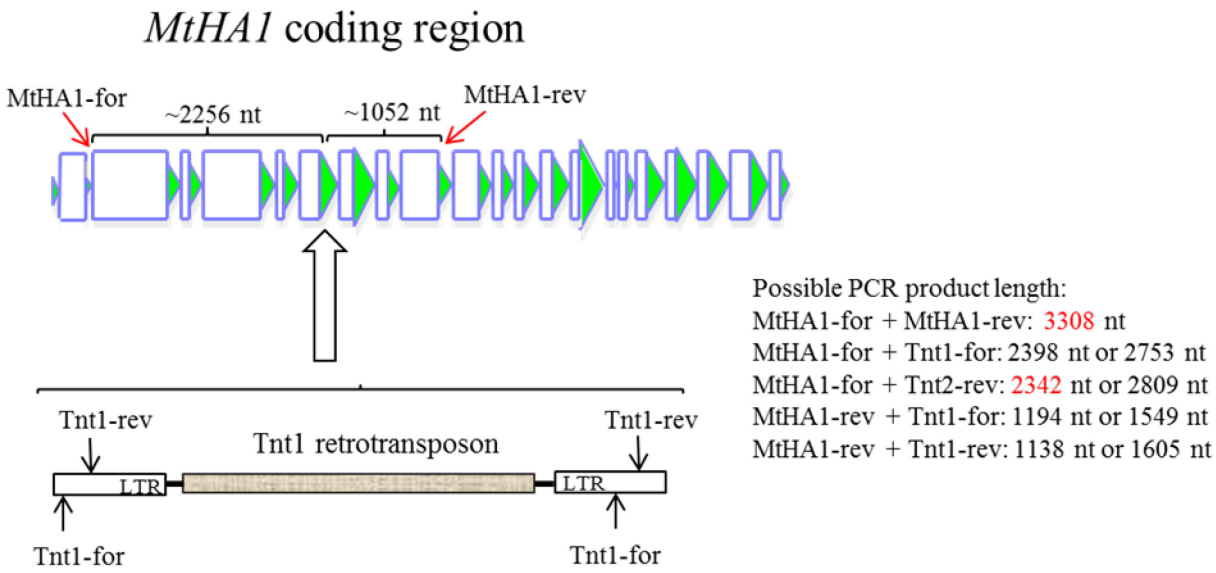

Figure 3.5-1 Diagram of retrotransposon *Tnt1* in *MtHA1* exon 7. Primer pair 1was located on exon 2 and exon 10 (red arrow) (white square represent introns while green arrow represent exons). Primer pair2 was located on the inverted repeat region of *Tnt1* retrotransposon. The possible length of PCR production was calculated based on *Tnt1* insertion position and the primer binding positions.

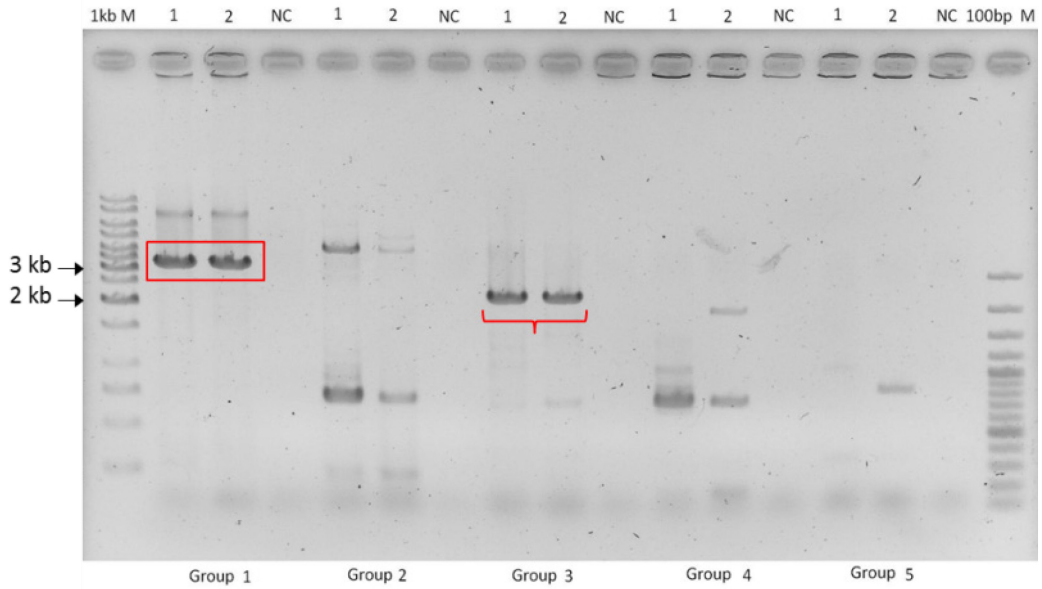

Figure 3.5-2 Gel electrophoresis of PCR productsobtained by using different primer groups and DNA extracts from two heterozygous plants as template. Group 1: MtHA1-for and MtHA1-rev; Group 2: MtHA1-for and Tnt1-for; Group3: MtHA1-for and Tnt1-rev; Group 4: MtHA1-rev and Tnt1-for; MtHA1-rev and Tnt1-rev; 1kb M: 1kb DNA ladder marker; 100bp M: 100bp DNA ladder marker; 1 and 2: DNA samples; NC: negative control. The PCR product of primer group 1 (red square) and primer group 3 (red bracket) showed clear and exclusive band matching the predicated length after gel electrophoresis.

3.5.2.2 Screening for*MtHA1 exon7::Tnt1* homozygous mutant and corresponding wild type plants

Based on a segregation of 1:2:1 (homozygous wild type : heterozygous : homozygous mutant), the probability is approximately 99% to detect at least one homozygous mutant among 16 individuals of the progeny after self-pollination ofa heterozygous plant (Clark and Carbon, 1976). Three generations of more than sixteen*M. truncatula* seedlings were cultivated and tested, but only heterozygous and homozygous wild type plants could be obtained based on the PCR detection described above (**Table3.5-1**). Based on the fact that some seedlings died at relative early stages in pot cultures, it was suggested that they might possess the homozygous mutant phenotype. Therefore germinated seeds were directly tested for their genotype by PCR. However, only heterozygotes and homozygous wild types were also detected in the seedling stage (**Table3.5-1**).

Table3.5-1*MtHA1*::exon7 genotypes. In each generation, seeds were collected from self-pollination of heterozygous plants of the previous generation. WT, wild type; MUT, mutant.

	Heterozygotes	WT homozygotes	MUT homozygotes	Dead
First generation	27	6	0	5
Second generation	13	4	0	5
Third generation	14	4	0	6
Germinated seeds	14	17	0	na

Note: na, not applicable

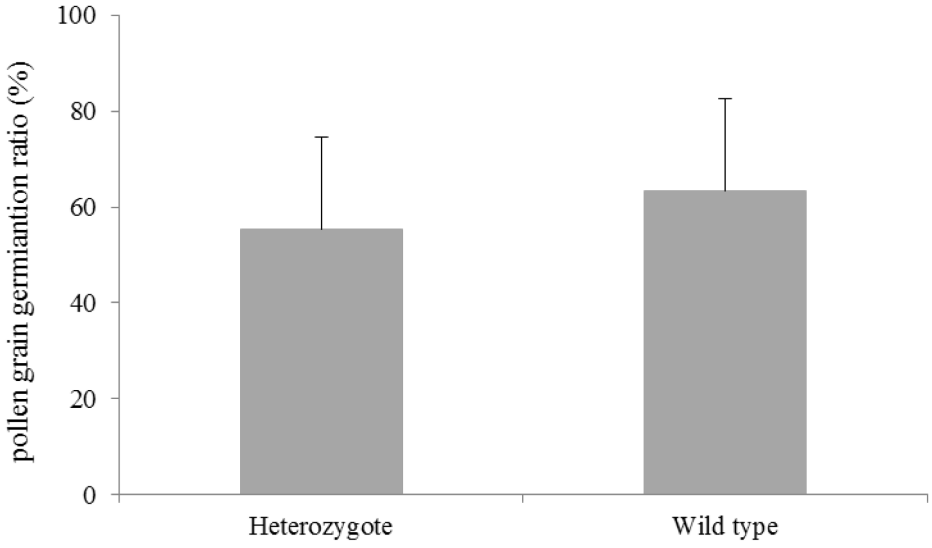

Figure 3.5-3 Germination ratio of pollen grains from each six*MtHA1*::exon7 heterozygous and the corresponding homozygous wild type plants. No significant difference was observed. Bars = standard deviations

3.5.2.3 Pollen grain germination

Because homozygous *MtHA1* exon7 mutant seeds could not be obtained, it was hypothesized that haploid mutant pollen grains in the flowers of heterozygous plants might not be viable. Therefore pollen grains from six homozygous wild type and six heterozygous plants were collected and tested for germ tube growth. However, no significant difference was observed between pollen grains of heterozygotes and homozygous wild type plants.

3.5.2.4 Function analysis of the *MtHA1 exon7::Tnt1* heterozygote plants

Based on the unsuccessful attempts to isolate a homozygous *MtHA1 exon::Tnt1*mutant, it was hypothesized that the function of *MtHA1* will be already reduced in heterozygous plants where*Tnt1*is only inserted in exon 7 of one allele of *MtHA1*. To test this hypothesis, heterozygous and the corresponding wild type plants were grown under phosphate limited condition and inoculated with *F. mosseae*. Four weeks after inoculation, plant growth and mycorrhization parameters, as well as phosphate content and gene expression patterns were compared between the heterozygotes and the wild type.

3.5.2.4.1 Plant growth

MtHA1exon7::Tnt1 heterozygotes and wild type plants were inoculated with *F. mosseae* and grown together with mock-inoculated plants under conditionsdescribed in the Materials and Methods section. Plant growth parameters were measured four weeks after inoculation (**Table 3.5-2**). Despite plant genotypes,inoculation of *F. mosseae* significantly improved plant growth parameters. The heterozygous plants had a larger biomass than wild type plants and this was significant for shoot and root fresh weight. Interactions between mycorrhization and genotype were not detected, but the genotype significantly affected MRI. MGD was not influenced by the genotype.

3.5.2.4.2 Mycorrhiza formation

Four weeks after inoculation, mycorrhizal parameters of wild type and heterozygous plants were assessed in stained root samples (**Table 3.5-3).** No mycorrhizal structures were observed in the mock-inoculated plants (data not shown). Infection frequency (F%), and arbuscule abundance (a% and A%) showed no difference between inoculated wild type and heterozygous plants, but mycorrhization intensity (m% and M%) of wild type plants was significantly higher than the values in heterozygous plants.

Table 3.5-2 Impact of mycorrhization on plant growth parameters of wild type and *MtHA1 exon7::Tnt1* heterozygotes plants. Average values of different parameters ± standard deviation are shown. Two-way ANOVA revealed no significant interactions between the factors genotype and mycorrhizal inoculation (myc) for all growth parameters. FW, fresh weight; DW, dry weight; MRI, mycorrhiza ratio increase; MGD, mycorrhiza growth dependency; WT, *M. truncatula* wild type; HET, *M. truncatula MtHA1 exon7::Tnt1* heterozygote; M, inoculated with *F. mosseae*; NM, without inoculation

treatments	shoot FW (g)	root FW (g)	shoot DW (g)	shoot/root	MRI	MGD (%)
WT NM	0.74±0.07	1.47±0.1	0.12±0.01	0.5±0.04	-	-
WT M	1.47±0.22	1.74±0.14	0.24±0.04	0.84±0.1	1.69±0.20	50.55±7.68
HET NM	0.9±0.11	1.68±0.1	0.14±0.02	0.53±0.04	-	-
HET M	1.63±0.13	2.03±0.15	0.26±0.03	0.81±0.04	1.51±0.12	45.08±6.38
genotype	s	s	ns	ns	s	ns
myc	s	s	s	s	na	na
genotype*myc	ns	ns	ns	ns	na	na

Note: s. significant difference or interaction at $P = 0.05$, $n = 4$; ns, no significant difference at $P = 0.05$ $n = 4$; na, not applicable.

Table 3.5-3 Mycorrhization parameters in wild type and *MtHA1 exon7::Tnt1* heterozygous plants. Average values of different parameters ± standard deviation are shown. In mock-inoculated samples, no mycorrhizal structures were observed. One-way ANOVA indicated significant effect of plant genotype on absolute mycorrhization intensity and relative mycorrhization intensity. WT, *M. truncatula* wild type; HET, *M. truncatula MtHA1 exon7::Tnt1* heterozygote; M, inoculated with *F. mosseae*; F%, infection frequency; M%, absolute mycorrhization intensity; m%, relative mycorrhization intensity; a%, relative arbuscule abundance; A%, absolute arbuscule abundance.

treatment	F%	M%	m%	a%	A%
WT M	85.85±6.04	53.99±5.46	62.83±3.49	30.33±12.52	16.85±8.17
HET M	87±6.83	42.02±3.12	48.35±2.33	21.92±6.2	9.14±2.16
genotype	ns	s	s	ns	ns

Note: s. significant difference at $P = 0.05$, $n = 4$; ns, no significant difference at $P = 0.05$ $n = 4$

3.5.2.4.3 Phosphorus uptake

Phosphorus (P) concentrations were measured in shoots of *F. mosseae*-colonizedplants and in corresponding controls four weeks after inoculation. Based on the data obtained and on the dry weight of shoots (**Table 3.5-2**), phosphorus uptake was calculated (**Figure 3.5-4**). Genotype and inoculation of *F. mosseae* did not affect shoot phosphorus concentration. Phosphorus uptake was significantly improved by mycorrhization, but the genotype showed no influence on this improvement.

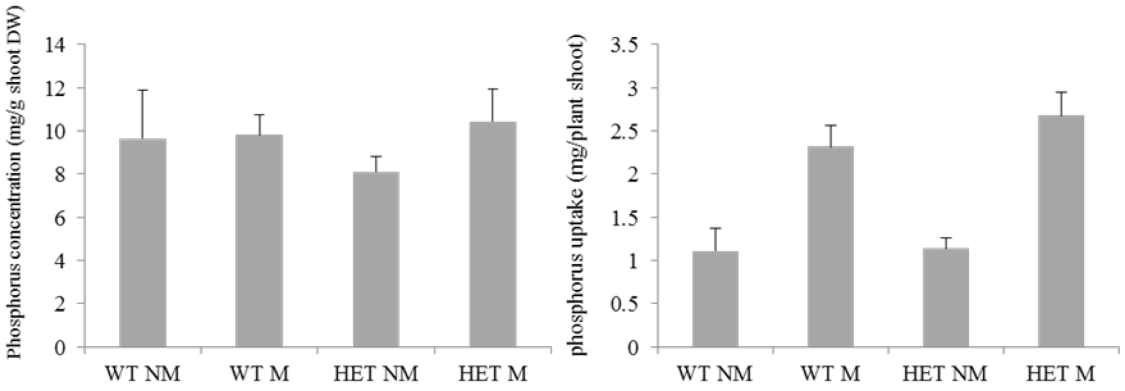

Figure 3.5-4 Influence of mycorrhization with *F. mosseae* on shoot phosphorus (P) concentration and uptake. Uptake were calculated in *M. truncatula*wild type and heterozygous plants based on measurements of phosphorus concentrations and shoot dry weights (P uptake = P concentration x shoot dry weight). Shown are mean values and standard deviations. Two-way ANOVA reveal no significant effect of genotype on phosphorus concentration and phosphorus uptake, while mycorrhization significantly improved phosphorus uptake but didnot affect phosphorus concentration. Interaction between the factors genotype and mycorrhization was not detected. WT, *M. truncatula* wild type; HET, *M. truncatula MtHA1 exon7::Tnt1* heterozygote; M, inoculated with *F. mosseae*; NM, without inoculation. Bars = standard deviations.

3.5.2.4.4 Relative gene expression

In order to test the hypothesis that the *Tnt1* insertion in one allele of *MtHA1* exon7 affects the expression of genes which present markers for mycorrhization, the RNA accumulation of four genes were studied by real-time RT-PCR (**Figure 3.5-5**). The expression of *FmTEF* as fungal housekeeping gene encoding a transcriptional elongation factor of *F. mosseae* should mirror the activity of the AM fungus. *MtPT4* and *MtBCP1*were selected as marker genes for arbuscule development (see **3.1.2.5**), while *MtHA1*with the primer pair located on exon 6 was analyzed in order to see, if there was any feedback mechanism in expression regulation. Inoculation of *F. mosseae* resulted in detection of *FmTEF* and significantly induces the expression of all three plant genes. Differences between the genotypes were, however, not detected (*MtHA1*, *MtPT4* and *MtBCP1*) or too slight for being significant (*FmTEF*).

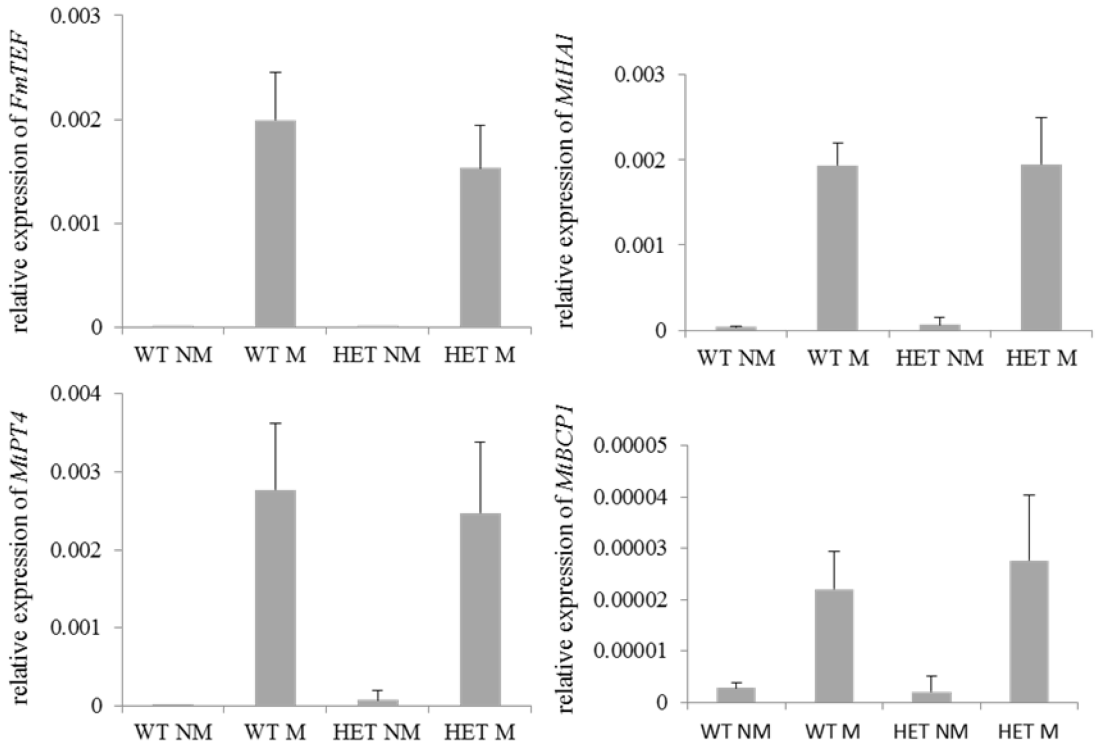

Figure 3.5-5 Gene expression level of *FmTEF*, *MtHA1*, *MtPT4* and *MtBCP1*. RNA was extracted from roots of wild type and *MtHA1 exon7::Tnt1* heterozygous plants inoculated or not with *F. mosseae*. Values obtained for the four genes were normalized with the values of *MtTEF1α* as reference gene. Two-way ANOVA indicated a significant effect of mycorrhization, but neither of the plant genotype nor any interaction. WT, *M. truncatula* wild type; HET, *M. truncatula MtHA1 exon7::Tnt1* heterozygote; M, inoculated with *F. mosseae*; NM, without inoculation. Bars = standard deviations.

3.5.3 Discussion

The small genome size (about 500 Mbp) and the availability of a facile transformation system make *M. truncatula* an ideal plant in generating large numbers of insertion mutants (Tadege et al., 2008). *Tnt1* used for *M. truncatula* mutagenesis is one of the few already characterized long terminal repeat (LTR) retrotransposons in plants. It is active during tissue culture, but stable during seed-to-seed propagation (d'Erfurth et al., 2003). This character of *Tnt1* allows the isolation of homozygotes from single gene mutations by self-pollination of heterozygous plants. As segregation of alleles from a single gene in *M. truncatula*, like *MtHA1*,should follow the second Mendelian rule: self-pollination of a heterozygote results in 25% homozygous wildtype, 50% heterozygotes and 25% homozygous mutant plants. Among 68 tested seedlings from a population of plants carrying the *MtHA1 exon7::Tnt1* allele, however, only heterozygotes (54) and homozygous wild type (14) plants were isolated. No homozygous mutant could be obtained. One possible explanation for the absence of homozygous mutant

plants could be that the total absence of a functional wild type allele in homozygous plants is lethal. Mutation of genes involved in different physiological functions can result in seedling lethality (Feldmann, 1991; Budziszewski et al., 2001; d'Erfurth et al., 2003). Seeds from these mutants can germinate but growth will be arrested and the seedlings will die. Based on the result that 16 seedlings died shortly after germination, 31 seeds from heterozygotes were germinated and directly used for genotype assessment. However again, no homozygous mutant was identified. This result excluded the possibility that the *Tnt1* insertion in exon7 of*MtHA1* leads to seedling lethality, but indicates that already no homozygous mutant seeds exist.

H^+-ATPases pump protons across plasma membranes in different plant tissues, for instance in leaves, in the epidermis orin the xylem (Palmgren, 2001). *NtAHA*encoding an H^+-ATPase in *Nicotiana tabacum*is specifically expressed during the development of pollen tubes(Certal et al. 2008). If this function is essential, mutation in such a gene would lead to pollination failure of haploid pollen grains carrying this allele. A heterozygous plant would then produce 50% vital and 50% non-functional pollen grains. Therefore pollen grains were collected from homozygous wild type and heterozygous flowers. However, the similar germination ratio indicated that the insertion of *Tnt1* in *MtHA1* exon7 did also not affect pollination. Unaffected pollination leaves as the next possible explanation that a function of the female gametophyte, the embryo sac, is affected. RT-PCR analysis of RNA extracted from flowers did not result in any amplified cDNA fragment (data not shown), but this could be due by a dilution effect, because the embryo sac presents only a very few cells. Alternative methods would be the analysis of gene expression by either laser microdissection of this tissue or by transgenic plants carrying promoter-reporter constructs. Another explanation could be that *Tnt1*, which has an average of 25 copies in *M. truncatula* mutant genomes (Tadege et al., 2008), is additionally inserted into a gene which is located on the same chromosome in close proximity to *MtHA1*, and which is important during seed formation as speculated in previous *M. truncatula* mutant segregation(d'Erfurth et al., 2003).

Gene dosage effect can be defined as that the copy number of a gene is correlated with the production of the corresponding protein and that reduced production of this protein influences the phenotype (Sano, 1984). In diploid plants, homozygous genotypes with two identical alleles of a particular gene are comparedwith the corresponding heterozygous genotype with two different alleles. This revealed that a number of genes exhibit an allele-dosage effect such

as *tb1* in maize, *fw2.2* in tomato, and *FLC* in Arabidopsis (Doebley et al., 1997; Michaels and Amasino, 1999; Frary et al., 2000). In order to test whether *MtHA1* shows such a dosage effect, the heterozygotes and wild type plants were inoculated with *F. mosseae*, and their growth, mycorrhization, phosphorus uptake and gene expression patterns werecompared. Although most parameters were not significantly affected, a slight dosage effect could be observed. Heterozygous plants showed less mycorrhization frequency (M% and m%) and mycorrhiza shoot/root ratio was reduced. The unaffected RNA accumulation may be explained by either the double expression of the normal allele to compensate the lost function of the allele with the*Tnt1* insertion or in the homozygous wild type plants only one allele is transcribed (Birchler et al., 2001).

3.6 Do jasmonates play a role in arbuscular mycorrhiza-induced local bioprotection of *Medicago truncatula*against root rot disease caused by *Aphanomyces euteiches*?

Hilou, A., Zhang, H., Franken, P., & Hause, B. (2014). Do jasmonates play a role in arbuscular mycorrhiza-induced local bioprotection of *Medicago truncatula* against root rot disease caused by *Aphanomyces euteiches*? *Mycorrhiza, 24(1),* 45-54.

- For this paper, I established the method to quantify the pathogen by qRT-PCR and carried out the experiment concerning the interaction of *F. mosseae* with the pathogen *Aphanomyces euteiches* (**Figure 2**).

3.7 Analysis of *MtHA1* function for mycorrhiza-induced resistance

3.7.1 Introduction

Aphanomyces euteiches Drechs, which belongs to the oomycetes, causes seedling and root rot disease of many legume crop plants and is the most severe soil borne pathogen of pea (*Pisum sativum* L.) in different countries (Gaulin et al., 2007). The infection cycle starts with the germination of oospores in soil which gives rise to numerous zoospores that encyst upon contacting the root surface.These cysts germinate and hyphae directly penetrate host cells at root tips from where root colonization starts. Finally antheridia fertilize oogonia and new oospores are formed inside the root. Upon root deceasing, these oospores serve as new inoculum (Kjøller and Rosendahl, 1998). The distinctive symptoms of this disease are honey-brown colored lesions inside the root cortex, and a watery rotting of root tissues (Colditz et al., 2004). *A. euteiches* can infect pea at any developmental stage. Early infection will result in plant collapse and total loss of yield, while late infection affects roots only partially and plants still produce pods with seeds (Gaulin et al., 2007). Inoculum of *A. euteiches* can survive in soil for more than 10 years and caninfect alternative hosts without displaying any symptoms. No effective strategyis applicable as no efficient fungicide and no fully resistant pea cultivar exist (Gaulin et al., 2007). The first evidence that arbuscular mycorrhizal fungi protect pea against*A. euteiches* was presented by Rosendahl (1985). Further studies confirmed these results, showing that mainly a pre-established mycorrhiza induces resistance against the pathogenwhile co-inoculation of AM fungi and *A. euteiches* didnot (Rosendahl, 1985; Bødker et al., 1998; Slezack et al., 1999 and 2000; Bødker et al., 2002). However, Thygesen et al. (2004) showed that such a co-inoculation can also reduce root rot in pea. In order to bypass the limitations caused by the large and complex *P. sativum* genome, the model plant *M. truncatula* was introduced into the research on legume- *A. euteiches* interaction (Nyamsuren et al.,2003). *M. truncatula* hasa much smaller genome which is totally sequenced and can be easily transformed. As shown before, inoculation by AM fungi is able to reduce the activity, the spread and the formation of *A. euteiches* oospores in *M. truncatula* roots (Colditz et al., 2005; Franken et al, 2007).

Previous studies indicated that mycorrhiza-induced resistance (MIR) against *A. euteiches* depends on the presence of functional arbuscules, becausesuppressionof arbuscule development by gibberellic acid application reduced the bioprotecting effect of an AM fungus against*A. euteiches* (Slezack et al., 2000).Based on limited truncated arbuscule formation in

MtHA1 mutants (result **3.1**,**3.2**, **3.3**), it was hypothesized that the resistance against *A. euteiches* will be reduced in such*M. truncatula* mutant plants. To test this hypothesis, *A. euteiches*-and/or *F. mosseae*-inoculated *MtHA1 exon8::Tnt1* homozygous mutant and corresponding wild type plants were compared concerning plant growth, mycorrhization and pathogen infection.

3.7.2 Results

3.7.2.1 Plant growth

MtHA1 exon8::Tnt1 mutant and the corresponding wild type seedlings were inoculated with *F. mosseae* during transplantation and grown together with mock-inoculated control plants for four weeks. Half of the plants were then inoculated with *A. euteiches* by applying zoospores to roots. Plant growth parameters were measured two weeks after pathogen infection (**Table 3.7-1**). Inoculation by the AM fungus did not affect shoot growth of both wild type and mutant plants, while its effect on root growth was converse for the two genotypes. Pathogen inoculation did not affect shoot or root growth, but significantly reduced MRI (mycorrhiza increased shoot/root ratio) in wild type and mutant plants.MGD(mycorrhiza growth dependency) was also affected by the pathogen by mitigating the negative or the positive mycorrhizal effect. A significant difference was, however, only detected between wild type and mutant plants not infected by *A. euteiches*.

3.7.2.2 Mycorrhizas formation

At the date of harvest (six weeks after inoculation with *F. mosseae* and two weeks after *A. euteiches* infection), mycorrhiza parameters were measured in trypan blue-stained roots (**Table 3.7-2**). No mycorrhizal structures were observed in mock-inoculated plants (data not shown). Plant genotype showed a significant influence on mycorrhiza formation as all mycorrhizal parameters of wild type plants were higher than in mutant plants. Inoculation of *A. euteiches* significantly reduced the absolute arbuscule abundance (A%) in wild type plants, but did not impact the other parameters. Similar effects were also observed for the other mycorrhization parameters, but differences were not significant.

3.7.2.3 Relative gene expression

In order to analyze the activity of the AM fungus and of the pathogen in plant roots, specific primers were designed to monitor gene expression by real-time RT-PCR (**Figure 3.7-1)**. *FmTEF* which codes for a transcriptional elongation factor of *F. mosseae* was used to quantify the activity of the AM fungus*F. mosseae* in plant roots, while the RNA accumulation ofthe5.8SrRNA of *A. euteiches*mirrored the activity of the pathogen (Hilou et al., 2013). *MtPT4*was used to quantify the number of functional arbuscules in plant roots (Harrison et al., 2002). *FmTEF* and *MtPT4*were only expressed in mycorrhizal roots, but the expression was much lower in mutant than in wild type plants. *A. euteiches* repressed RNA accumulation of both genes in the wild type, but only of *MtPT4* in the mutant. *A. euteiches*5.8S rRNAaccumulates in non-mycorrhizal plants and this accumulation was reduced in roots pre-inoculated by*F. mosseae*. This reduction of pathogen RNA accumulation showed no significant difference between wild type and mutant plants (**Figure 3.7-1)**.

Table 3.7-1 Impact of inoculation of *F. mosseae* and *A. euteiches* on growth parameters of wild type and *MtHA1 exon8::Tnt1* mutant plants. Average values of growth parameters ± standard deviations are shown. Inoculation of *A. euteiches* (Ae) had significant impact on mycorrhiza ratio increase (MRI). Interaction of plant genotype (geno) and mycorrhization (myc) was significant for root fresh weight (FW), while interaction of plant genotype (geno) and inoculation of *A. euteiches* (Ae) was significant for mycorrhiza growth dependency (MGD). Values with different letter indicate significant differences (LSD-test $P = 0.05$, $n = 4$). WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; M, inoculated with *F. mosseae*; A, inoculated with *A. euteiches*; MA, inoculated with *F. mosseae* and *A. euteiches*; NM without inoculation.

treatment	shoot FW (g)	root FW (g)	shoot DW (g)	shoot/root	MRI	MGD (%)
WT NM	2.11±0.61	2.05±0.57	0.29±0.08	1.03±0.03	-	-
WT M	1.98±0.30	1.40±0.59	0.25±0.05	1.61±0.63	1.57±0.62	-0.19±0.27 b
WT A	1.71±0.44	1.85±0.11	0.25±0.06	0.92±0.22	-	-
WT MA	1.81±0.35	1.74±0.21	0.25±0.05	1.04±0.09	1.13±0.10	-0.04±0.21 ab
MUT NM	1.87±0.9	1.87±0.69	0.26±0.13	1.02±0.11	-	-
MUT M	2.59±0.35	2.14±0.36	0.33±0.04	1.22±0.07	1.19±0.07	0.20±0.09 a
MUT A	2.06±0.38	1.74±0.22	0.31±0.04	1.23±0.40	-	-
MUT MA	2.15±0.48	1.98±0.17	0.29±0.05	1.08±0.17	0.88±0.14	-0.11±0.24 ab
geno	ns	ns	ns	ns	ns	ns
myc	ns	ns	ns	ns	na	na
Ae	ns	ns	ns	ns	s	ns
geno*myc	ns	s	ns	ns	na	na
geno*Ae	ns	ns	ns	ns	ns	s
myc*Ae	ns	ns	ns	ns	na	na
geno*myc*Ae	ns	ns	ns	ns	na	na

Note: s, significant influence or interaction at $P = 0.05$, $n = 4$; ns, no significant difference at $P = 0.05$, $n = 4$; na, not applicable.

Table 3.7-2 Impact of plant genotype and *A. euteiches* infection on mycorrhization parameters. Average values of parameters ± standard deviation are shown. Two-way ANOVA indicated that plant genotypes had a significant effect on all mycorrhiza parameters while pathogen inoculation only affects absolute arbuscule abundance. No significant interaction between plant genotype and pathogen inoculation was observed. As pathogen inoculation had a significant effect on absolute arbuscule abundance, t-tests were carried out and significant difference in the same genotype was indicated by asterisks. WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; M, inoculated with *F. mosseae*; A, inoculated with *A. euteiches*; MA, inoculated with *F. mosseae* and *A. euteiches,* F%, infection frequency; M%, absolute mycorrhization intensity; m%, relative mycorrhization intensity; a% relative arbuscule abundance; A%, absolute arbuscule abundance.

treatment	F%	M%	m%	a%	A%
WT M	65.68±14.19	23.49±8.84	35.19±7.80	52.81±10.71	12.39±4.7
WT MA	54.36±14.78	15.48±5.21	28.31±4.21	40.89±4.68	6.15±1.25 *
MUT M	39.23±10.99	7.72±3.89	19.11±4.98	39.36±9.13	3.13±2.10
MUT MA	39.42±10.99	7.44±3.95	17.67±6.97	35.19±3.40	2.57±1.25
geno	s	s	s	s	s
Ae	ns	ns	ns	ns	s
geno*Ae	ns	ns	ns	ns	ns

Note: s, significant influence or interaction at $P = 0.05$, $n = 4$; ns, no significant difference at $P = 0.05$, $n = 4$, *, significant at $P = 0.05$, $n = 4$(t-test).

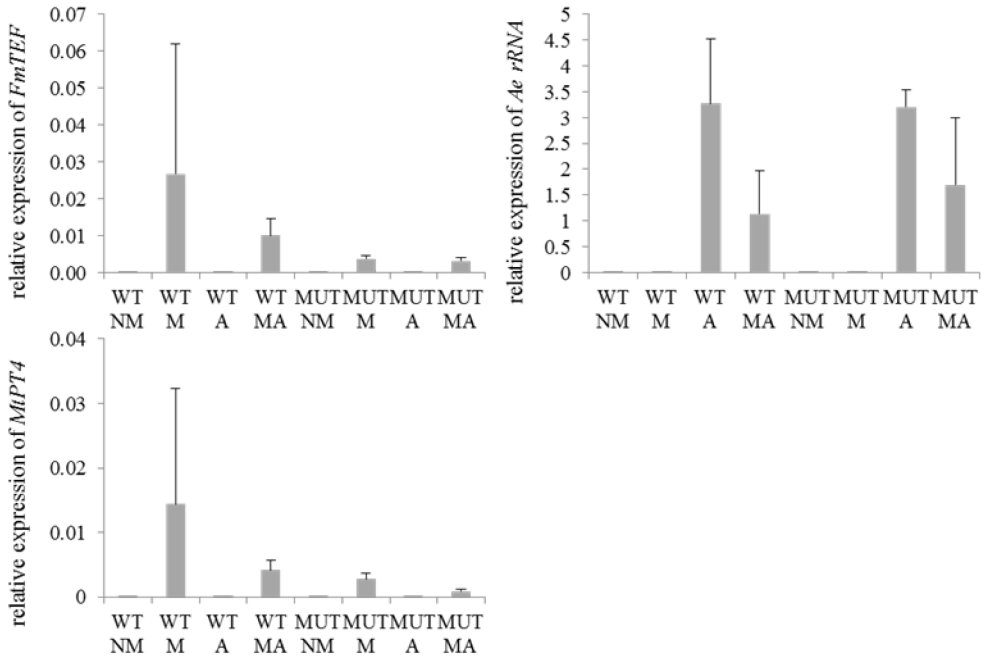

Figure 3.7-1 Relative expression of *FmTEF*, *A. euteiches*5.8S rRNA, and *MtPT4*. RNA was extracted from roots of different treated plants. Values obtained for these genes were normalized with the value of *MtTEF1α* as reference gene. Three-way ANOVA indicated significant effect of *F. mosseae*inoculation on expression of *A. euteiches*5.8S rRNA and *MtPT4,* while pathogen inoculation and plant genotype did not affect the expression of *FmTEF* and *MtPT4*. WT, *M. truncatula* wild type; MUT, *M. truncatula MtHA1 exon8::Tnt1* mutant; M,

inoculated with *F. mosseae*; A, inoculated with *A. euteiches*; MA, inoculated with *F. mosseae* and *A. euteiches*; NM, without inoculation. Bars = standard deviations

3.7.3 Discussion

When*A. euteiches* invades plant root systems, it initially reduces normal root functions as e.g.nutrient uptake.As a devastating pathogen, it can cause finally root rot and plant death (Gaulin et al., 2007). In previous studies, different methods were used to quantify the pathogen inside root systems. At the beginning, the quantification was based on thedisease symptoms (Rao et al., 1985). This method was, however, unreliable, because dependent on the plant species and genotype and on the stage of infection, the pathogen could colonize the root without showing any symptoms (Franken et al., 2007). Other methods based on enzyme activities, particular proteins or other compounds were applied in pathogen quantification e. g. oospore staining with a test for alkaline phosphatase activity, ELISA, and fatty acid detection (Kjøller and Rosendahl, 1998; Slezack et al., 1999;Larsen et al., 2000; Larsen and Bødker, 2001). With the development of specific primer pairs, real-time RT-PCR was applied for pathogen detection and quantification and this is nowadays seen as a reliable, effective and time-saving method (Vandemark et al., 2002; Vandemark and Barker, 2003; Vandemark and Grünwald, 2005). In a recent study, usage of specifically designed primers located in the genes for5.8S rRNA and for a sterol C24 reductase resulted in rapid and reliable detection and quantification of *A. euteiches* in *M. truncatula* roots (Hilou et al., 2013). In the current study, detection of *A. euteiches* was conducted two weeks after inoculation. Differencesin*A. euteiches* infection between mycorrhizal and non-mycorrhizal plants were already significant while the typical honey-brown colored lesion in root cortex were still only subtle.

Based on the type of interaction with plants, pathogens can be divided into necrotrophs that kill host and feed on the content, and biotrophs that need living hosts to obtain nutrients and to complete their life cycle. When plant encounter different types of pathogens, different defense reactions are activated (Dangl and Jones, 2001; Kunkel and Brooks, 2002; van Loop et al., 2006). In general, salicylic acid (SA) mediatesdefense reactions against biotrophs, while jasmonate (JA) and ethylene (ET) dependent responses act against necrotrophs (McDowell and Dangl, 2000). Defined as a hemibiotroph (Franken et al., 2007), the defense reaction that are elicited by *A. euteiches* could be much more complicated and might change during time (Colditz et al., 2005). As a widely formed symbiosis, arbuscular mycorrhizas play an important role in pathogen resistance (Pozo and Azcon-Aguilar, 2007). The so-called mycorrhiza-induce resistance (MIR) can help plants to resist pathogens in different ways

(Jung et al., 2012). As already mentioned, one important function of AM is the nutrient status improvement especially based on phosphate supply (Smith and Read, 2008). In previous studies, the improvement of phosphate in *MtHA1* mutant plants was reduced as well as the expression ofthe mycorrhiza-specific phosphate transporter gene*MtPT4* (result **3.1**, **3.2**). In the current study, reduced expression of *MtPT4* in mutant plants representing the reduced nutrient improvement by the AM symbiosis, did not compromise the resistance against *A. euteiches*. This implies that phosphate status of *M. truncatula* is not the reason for changes in resistance or susceptibility. This goes along with the finding in the interaction between *G. intraradices* and *A. euteiches* in pea, where also increased phosphate levels did not explainmycorrhiza-induced resistance (Bødker et al., 1998). During the interaction between AM fungi and a bacterial pathogen in *M. truncatula*, it was also shown that the increased nutrient status is not the reason for improved resistance (Liu et al., 2007). With limited space in the rhizosphere, the AM fungi compete with pathogens for infection sites of the host root (Azcón-Aguilar and Barea, 1997; Wehner et al., 2009). Lowered mycorrhizal colonization in mutant plants still reduced the pathogen infection implying that infection site competition was not the case in this study. Competition of nutrient could be another explanation of AM fungi induced bioprotection (Azcón-Aguilar and Barea, 1997). Inwild type plants, the biomass of both *A. euteiches* and *F. mosseae* was reduced in co-inoculated roots where the reduced absolute arbuscule abundance might be interpreted as a response of *F. mosseae* to plant carbohydrates reduction (Larsen and Bødker, 2001). But the nutrient competition is not applicable in mutant plants as the reduced *A. euteiches* infection was coupled with non-influenced *F. mosseae* colonization.

Priming that sets plant in an ‘alert’ state in which the defenses are not actively expressed but respond to an attack faster and/or stronger, is one efficient and energy-saving strategywhich AM fungi might use for inducing pathogen resistance (Jung et al., 2012; Pastor et al., 2012). If this is the case, fully developed arbuscules seems not to be crucial for this function: although arbuscules in mutant plants were truncated (result **3.1**, **3.2**), the mycorrhizal reduction of pathogen activity mirrored by *A. euteiches*5.8SrRNA accumulation showed no difference between mutant and wild type plants. The presence of truncated arbuscules seems to be sufficient for MIR against the pathogen. AM fungal induced priming was proposedto depend on a functional JA signaling pathway and, wound-induced or exogenous applied JA increased the resistance against*A. euteiches* (Landgraf et al., 2012). JA biosynthesis isincreased in mycorrhizal roots(Vierheilig, 2004) and the site of the AM-induced synthesis is

the fully developed arbuscule (Hause et al., 2007). JA did, however, not play a major role in AM-induced local bioprotection against *A. euteiches* (Hilou et al., 2013). It is then reasonable to speculate that mycorrhiza-induced host plant resistance requires the intraradical development of AM fungi, but fully functional arbuscules are not necessary, because JA biosynthesis is not a prerequisite for MIR.

3.8 Local and systemic interaction between the arbuscular mycorrhizal fungus *Funneliformis mosseae* and the root pathogen *Aphanomyces euteiches* in *Medicago truncatula*

Zhang, H., & Franken, P. (2013). Local and systemic interaction between the arbuscular mycorrhizal fungus *Funneliformis mosseae* and the root pathogen *Aphanomyces euteiches* in *Medicago truncatula. Mycorrhiza,* DOI 10.1007/s00572-013-0553-4.

- This part of result is published on*Mycorrhiza*. All experiment was carried out by me.

4 General discussion

Based on fossil records, the existence of AM fungi can be traced back to 400 million years ago when primitive plants left water and started to colonize land (Remy et al., 1994; Brundrett, 2002). From that time, AM fungi assisted plants to increase mineral nutrient acquirement due to their hyphae,which intensively explore the surrounding soil, while receiving carbohydrates to enable their metabolomic activities and to complete their live cycle (Smith and Read, 2008). This nutrient exchange is the basis for the symbiosis and, interruption of this process will to different extent inhibit the formation of AM (Maeda et al., 2006; Javot et al., 2007b; Baier et al., 2010; Helber et al., 2011). Plants need large amounts of mineral nutrients such as phosphate, but most of this phosphate is presented inaccessible in soils (Bieleski, 1973). Unlike some non-mycorrhizal plants which form massive lateral roots or particular root structures to explore soils, mycorrhizal plants employed AM fungi for soil exploration and developed the mycorrhizal nutrient pathway (Smith et al., 2003; Pérez-Torres et al., 2008; Hodge, 2009). In case of the well-studied phosphate uptake, plant express specific transporters belonging to the *Pht1* family to acquire the nutrient from AM fungi (Harrison et al., 2002; Paszkowski et al., 2002; Maeda et al., 2006). Silencing of the specifically induced phosphate transporter gene e.g. in *M. truncatula* (*MtPT4*) that leads to arrest of the AM symbiosis, indicated the importance of the mycorrhizal phosphate uptake pathway for maintaining the relationship (Javot et al., 2007b). In a normal AM symbiosis, development and senescence of arbuscules, which is the site for nutrient transportation, in one root cell does not influence the fate of the arbuscules in neighboring cells (Kobae et al., 2010). The systemically inhibited symbiotic colonization of *MtPT4*-silenced plants implies that the phosphate transfer plays an important role for the overall communication between plants and AM fungi. Hence this inhibition seemed to be an actively controlled process instead of simple carbohydrate depletion. This view was supported by the finding that support of the AM fungus by a nursery plant did not result in colonization of the *MtPT4* mutant plant (Javot et al., 2011).

In the current study, mutation of an AM specifically induced proton pumping gene (*MtHA1*) led to reduced AM colonization with truncated arbuscules. Unlike in *MtPT4* silenced plants, the interaction still showed some symbiotic activity because the plants still obtained benefit such as promoted phosphate uptake (result **3.1**). The limited number of arbuscules and phosphate uptake was similar with the result obtained in*L. japonicus* plants, in which the AM-inducible phosphate transporter (non-orthologous to *MtPT4*) was silenced, but AM fungal

development was only moderately reduced (Maeda et al., 2006). In case of MtHA1, a residual proton gradient might be still formed by functional redundancy based on the activity of other root expressed plant proton-pumping ATPases as MtHA2 or MtHA3 (Krajinski et al., 2002) or on a fungal ATPase-encoding gene like *PMA1* or *HA5* (Requena et al., 2003). Alternatively, uptake of phosphate in mycorrhizal plants could be increased based on a mechanism independent of the formation of functional arbuscules (Ezawa et al., 2005; Li et al., 2012).

With the purpose of verifying the results obtained with the mutant where the exon8 of the gene was affected, a second independent mutant was tried to isolate by obtaining homozygous plants from a heterozygote where the *Tnt1* transposon was inserted in exon 7 of one *MtHA1* allele (result **3.5**). However, the corresponding homozygous plants could not be obtained. Because pollen grain germination was not abolished it was assumed that the mutation affected maternal functions of embryogenesis. Further analysis is needed to find out, if this is based on *MtHA1* itself which is unlikely because expression at this stage could not be detected. More probable a gene on the same chromosome in close proximity to *MtHA1* was interrupted by another *Tnt1* insertion and this gene plays an important role for the female gametophyte. This possible character makes the heterozygous plants to potential candidates for studying maternal controlled embryogenesis (Grossniklaus et al., 1998). However, inoculation of the heterozygous plants already showed a small but significant effect, as mycorrhization was slightly reduced. This confirms that mutating *MtHA1* leads to reduced colonization of roots by AM fungi.

AM fungi can supply plants with mineral nutrients, but when plants have access to large amounts of phosphate, limited formation and function of the symbiosis were observed (Breuillin et al., 2010). This was confirmed by the current analysis. When *M. truncatula* wild type plants were fertilized with optimal phosphate concentration, colonization was reduced and accompanied with lower levels of RNA accumulation of the three analyzed arbuscule specific genes. Combining the importance of phosphate in maintaining symbiosis and the reduced expression of phosphate transporter genes prior to reduced colonization, the limited AM fungal development under high phosphate fertilization is very probably the consequence (instead of the reason) of the limited phosphate uptake via the mycorrhizal pathway (Javot et al., 2007a; Breuillin et al., 2010). Phosphate uptake was most likely not based any more on the mycorrhizal but on the direct uptake pathway via root hairs and root epidermis (Result **3.2**). Mutation of *MtHA1* inhibited the mycorrhizal pathway of phosphate uptake. The

variation of phosphate concentration in fertilizer affected no longer mycorrhizal phosphate uptake and resulted in the constant low level of AM fungal development and abnormal arbuscules in mutant plants. Similar to high phosphate-fertilization, in plants that form AM in part of their roots, subsequent colonization of AM fungi will be limited and the overall mycorrhization will be maintained at a certain level. This phenomenon is called auto-regulation of mycorrhization (Vierheilig, 2004a). Controlling energy expenses was proposed to be the rationale behind auto-regulation and, phytohormones were later discovered to be involved (Vierheilig, 2004b; Meixner et al., 2005). If the same or different mechanisms are underlying auto-regulation, inhibition by highly available phosphate or by silencing of genes involved in the mycorrhizal phosphate uptake needs further investigations.

Due to the obligate biotrophic character, early attempts to axenically cultivate AM fungi failed (Hepper, 1984). Later it was demonstrated that the exclusive biosynthesis of 16-carbon fatty acids in intraradical hyphae was the reason, although AM fungi may uptake sugars by intra- and extraradical hyphae (Bago et al., 2000; Trepanier et al. 2005; Helber et al., 2011). It was suggested that the exchange of carbohydrates and phosphate is coupled in mycorrhizal plants (Bücking and Shachar-Hill, 2005). Such interdependency was furthermore demonstrated in a study where both, plants and AM fungi can reward a more ‘co-operative’ partner with enhanced delivery of the respective nutrients (Kiers et al., 2011). Some AM fungi can be less ‘co-operative’ and exhibit a parasitical way of life under certain circumstance by reduce (instead of promote) host plant growth, and consequently plant inhibited fungal extension inside root (Johnson et al., 1997). In the current study, *M. truncatula* plant limited the development of *G. rosea* that form *Paris*-type mycorrhiza with strong carbon demand, and mutation of *MtHA1* diminished the arbusculated hyphal coils in analogy to the truncated *Arum*-type arbuscules (Result **3.3**). Although arbusculated coils were suggested to play the same role as arbuscules (Dickson, 2004), uptake of phosphate relied on direct pathway instead of mycorrhizal pathway at the parasitic stage of *Paris*-type mycorrhization may result in the similar mycorrhizal parameters in *MtHA1* wild type and mutant plants (Burleigh et al., 2002; Smith et al., 2003). For which reason and to what extent the plant maintainsa parasitic mycorrhizal interaction was uncertain, but it is interesting to note firstly that AM fungi may be nutrient competitors as they can accumulate seven times more phosphate when the carbohydrate from root was halved (Hammer et al., 2011), and secondly that the parasitical life of AM fungi does not last a long period (Bethlenfalvay et al., 1982; Lerat et al., 2003).

Time course studies covering the parasitic and beneficial phases could shed light on the role of *MtHA1* in the*Paris*-type *G. rosea/M. truncatula* interaction.

Unlike AM that can be formed with more than 80% territorial plants and have a long history, RNS can be only traced back to about 100 million years and are found within a relatively limited number of plant species (Kistner and Parniske, 2002). Although both symbioses are formed with different types of microbes and have different functions and morphology, developing evidences are now supporting the idea that these two symbioses share a similar pathway which RNS had adopted from the AM (Parniske, 2008). From the initial signal perception to finally microbe accommodation and function, many genes were identified to manipulate the resembling procedure (see general introduction). Silencing of these genes influences not only AM but also RNS (Murray et al., 2011; Ivanov et al., 2012). In the current study, the unaffected formation and function of RNS in *MtHA1* silenced plants implied that *MtHA1* is not one of the genes (e.g. *MtVAMP72d/e*) involved in late stages of both symbioses (Result **3.4**). In the study of Maeda et al. (2006), silencing of *LjPT3* did not affect RNS development, but induced nodule necrosis when AM fungi and rhizobia were co-inoculated. This result indicated that plant may develop the same auto-regulation for both symbioses, although only AM was malfunctioned. Auto-regulation of nodulation was described in 1990s and later this regulation was observed also during AM development (Caetano-Anolles and Gresshoff, 1991; Vierheilig, 2004a). This regulation was later reported between AM and RNS (Catford et al., 2003, 2006). In a recent study, the relieved phenotype of mycorrhization in *MtPT4* mutant plants under phosphate- and nitrogen-deprived conditions indicated the importance of both nutrients in the common regulation (Javot et al., 2011). Combined with the discovery of the E3 ubiquitin ligase in crosstalk of phosphate and nitrogen signaling pathway (Kant et al., 2011), the common regulatory mechanism can be speculated to control symbiosis by monitoring the nutrient uptake. Co-inoculation of AM fungi and rhizobia in *MtHA1* mutant plants may provide further evidence in understanding the auto-regulation in *M. truncatula*.

Separated from the former genus *Glomus* (Schüßler and Walker, 2010), *F. mosseae* and *R. irregularis* were the most used species in the study of AM fungi. The influence of these two AM fungi on host plant was quite different as only a small part of regulated genes in *M. truncatula* overlapped (Hohnjec et al., 2005). For instance, *R. irregularis*colonization suppresses five phosphate transporter genes while *F. mosseae*colonization only suppresses

only two of them (Grunwald et al., 2009). The spread of *R. irregularis* in host plant roots is usually faster than the colonization of other AM fungi and the ability of phosphate supply is also stronger (Alkan et al., 2006; Smith et al., 2003, 2004). In the current study, the colonization of *R. irregularis* was also faster than *F. mosseae* under different phosphate condition in wild type plants (Result **3.2**). Mutation of *MtHA1* suppressed fungal development and resulted in truncated arbuscules for both AM fungi, but still the mycorrhizal parameters of *R. irregularis* were higher than those of *F. mosseae*. Based on the study of Kiers et al. (2011) that host plants may reward co-operative AM fungus supplying more phosphate with more carbohydrates, the faster development of *R. irregularis* in both genotypes implies a higher exchange of nutrients as for *F. mosseae* even if *MtHA1* is mutated. Besidesthe difference in speed of development, both AM fungi differed in helping host plants to resist adverse situations. Under drought, salinity and heavy metalstress, the promotion of host plant development by*F. mosseae* was better than by *R. irregularis* (Ruiz-Lozano et al., 1995; Daei et al., 2009; Christophersen et al., 2012). In the interactions between host plants and pathogens, the bioprotective effect of *F. mosseae* was also much efficient than of*R. irregularis* (Habte et al., 1999; Pozo et al., 2002; Hayek et al., 2012). A similar bioprotective effect of *F. mosseae* and *R. irregularis* was, however, observed in the *M. truncatula- A. euteiches*pathosystem (Hilou et al., 2013). The different identity of host plants, AM fungi and pathogens may be the reason for the divergent results and needs further systematic investigations.

Besides improved plant nutrition, another important function of AM is to induce resistance and/or tolerance against biotic and abiotic stress (Jeffries et al., 2003; Gianinazzi et al., 2010). Unlike the limited number of adverse abiotic conditions, a huge variety of biotic threads by different microbes, by insects and by parasitical plants exist and it is well documented that the interaction of the plants with these biotic threads are affected by AM (Whipps, 2004; Hartley and Gange, 2009; López-Ráez et al., 2009). Concerning the oomycete *A. euteiches,* a devastating pathogen for economically important legumes (pea and alfalfa), no resistant cultivar or applicable fungicide could be yet identified (Levenfors et al., 2003; Gaulin et al., 2007), but a control effect of AM was discovered (Rosendahl et al., 1985). In further studies, it was confirmed that only a pre-established AM induces resistance against *A. euteiches*, while in mutant plants, where AM fungi did not colonize the roots beyond the hyphopodium stage or developed only very few arbuscules, did not show mycorrhiza-induced resistance (MIR) (Slezack et al., 2000). In the current study, limited mycorrhization with truncated arbuscules

in *MtHA1* mutant plants did not result in a reduced protective effect against *A. euteiches* (Result **3.7**). Compared with the study of Slezack et al. (2000), in which the formation of arbuscules and the resistance against *A. euteiches* was both eliminated by exogenous application of gibberellic acid (GA), it is likely that a threshold level of developing arbuscules exists forMIR, while fine branches and corresponding functions of arbuscules are not needed. Although GA acts antagonistically towards the JA signaling pathway (Robert-Seilaniantz et al., 2007), the influenced JA signaling was not the reason of the eliminated resistance in the study of Slezack et al. (2000), because the biosynthesis of JA was confirmed to be not a prerequisite for MIR (Hilou et al., 2013). The result of the experiment proved in addition that increased phosphate nutrient is not the reason for the protective effect (Bødker et al., 1998; Liu et al., 2007). Competitionfor carbohydrates and/or for space, however, cannot be excluded as basis for MIR (Azcón-Aguilar and Barea, 1997; Wehner et al., 2009).

Priming is an efficient way for induced resistance because defense reactions are highly energy consuming (Conrath et al., 2006; Azcon-Aguilar, 2007; Bolton, 2009). In order to confine biotrophic pathogens, plants usually activate SA-dependent defense reactions (Glazebrook, 2005). With the character and behavior resembling biotrophic pathogens, root infection by AM fungi is accompanied by an early induction of an SA-dependent responses, but this is only transient and later repressed (García-Garrido and Ocampo, 2002; Güimil et al., 2005; Paszkowski, 2006). Plants constantly producing SA or exogenous application of SA usually exhibit a restricted AM fungal colonization (Blilou et al., 1999; Blilou et al., 2000; Herrera Medina et al., 2003). As a phytohormone with antagonistic effects to SA, JA biosynthesis was observed to be induced by AM fungi at late stages (Hause et al., 2002; Vierheilig, 2004a; Pieterse et al., 2009). AM fungal induced priming against different pathogens starts from the colonized root areas (Benhamou et al., 1994; Yao et al., 2003; Lee et al., 2005) and later reaches the whole root system (Cordier et al., 1998; Li et al., 2006; Hao et al., 2012; Hayek doctor thesis) and even the plant shoots (Pozo et al., 2009; Jung et al., 2012). AM fungal-induced priming is believed to depend on JA signaling as the rhizobacteria-induced priming (van Wees et al., 2008). If priming exists in the bioprotection of mycorrhizal *M. truncatula* plants against *A. euteiches*, it does, however, not depend on JA signaling as over-expression or knock-down of a key gene in JA biosynthesis did not affect MIR (Hilou et al., 2013).

Because it was difficult to separate direct from indirect effects in MIR, the infection of *A. euteiches* was monitored in a split root system with or without the AM fungus *F. mosseae* in

the distal root part (result **3.8**). Unlike expected, AM fungal colonization increased the susceptibility instead of resistance to *A. euteiches* infection. Based on the analysis of soluble sugars, common source competition was excluded from the mycorrhiza-induced resistance or susceptibility in the current study. When compared both experimental systems the activity of *A. euteiches* was ten times less in split rootsthan in pot cultures independent of mycorrhizal colonization, and was accompanied by highly induced expression of genes involved in plant responses to pathogens. It was reasonable to suggest that the stress during split root cultivation also primed plants to resist the pathogen.Inoculation with the AM fungus relieved the stress imposed to the plants and resulted in decreased defense genes expression and in consequence inincreased pathogen infection. In pot culture experiment, the AM fungus *F. mosseae* induced the resistance against *A. euteiches* andthe pathogen inhibited vice versa the development of the AM fungus. In consequence AM fungal-induced phytohormone levels were also reduced. Co-inoculation reduced the SA level which may negatively affect the JA/ET signaling pathways even if JA and ET levels were not modified. How the priming through JA/ET signaling pathway via suppression of its opposite SA signaling pathway fits to the finding that modulating the expression of a gene for an enzyme involved in JA biosynthesis did not affect MIR (Hilou et al., 2013) needs further investigations.

5 Concluding remarks and outlooks

In the first part of the current dissertation, the role of a proton pump-encoding gene,*MtHA1*,for the AM symbiosis in *M. truncatula*was investigated. Insertion of the transposon *Tnt1* in exon 8 of *MtHA1* results in a shortened transcript which can be only translated into a protein missing an important domain for its function. In the beginning of the interaction (two weeks after inoculation with the AM fungus *F. mosseae*)arbuscules start to develop as in wild type plants. The mutated MtHA1 protein, however, cannot provide the electrochemical gradient necessary for the transport of phosphate. Because the plant cannot receive sufficient phosphate via the mycorrhizal pathway, it does not allow the formation of fungal arbuscule branches and only truncated arbuscules are formed. *MtBCP1* still shows normal expression because the encoded protein can be located in the arbuscule trunk (Pumplin and Harrison, 2009). The transcription of *MtPT4* and *MtHA1*is, however, reduced via a feedback mechanism as response to the absence of the membrane around the fine branches where the corresponding proteins normally fulfill their functions(Pumplin and Harrison, 2009; Pumplin et al., 2012). Mutant plants six weeks after inoculation had taken up much less phosphate because the mycorrhizal pathway has not worked properly. This low phosphate gain let the plant provide less carbohydrates for the AM fungus and overall hyphal development of the symbiotic partner is reduced. This pattern with slight modifications can be also observed in interactions with the fast-colonizer *R. irregularis* and the *Paris*-type arbuscule-forming *G. rosea*. Higher phosphate fertilization levels let the plant reduce the mycorrhizal uptake pathway including *MtHA1* expression. In this case the mutation of the gene did not exert any additional effect on fungal colonization. In the root nodule symbiosis between *M. truncatula* and *S. meliloti*, mutation of *MtHA1* affected neither nodule formation nor nitrogen uptake. This indicates that *MtHA1*does not encode the proton pump necessary for protonation of NH_3.

In the split root system used for the current dissertation, plants seems to be stressed which led to increased expression of a number of defense-related genes and a reduced colonization of the roots by the pathogen *A. euteiches*. Inoculation of the system with the AM fungus reduced the stress, the defense-related genes where not so highly induced anymore and the roots were more susceptible for the pathogen. This phenomenon seemed to be mainly based on the

phytohormone salicylic acid. In pot cultures the AM fungus protected the plant against the pathogen and this protection was independent of high mycorrhizal colonization levels and fully developed arbuscules. In contrast to the split root system, salicylic acid seemed to play a negative role in defense against *A. euteiches* in pot cultures.

In the current study, mutation of *MtHA1* results in limited AM fungal development, truncated arbuscules and reduced phosphate supply of the plant. This demonstrates the role of the gene *MtHA1*for mycorrhiza, but a number of questions could not be answered, e. g. whether the MtHA1 protein is specifically located on membrane surrounding arbuscular branches like MtPT4 (Pumplin and Harrison, 2009).Localization of fluorescence from mycorrhizal root fragments containing fusion of GFP gene with *MtHA1* will probably provide an answer to this question. Furthercomparison of MtHA1 with MtPT4 localization may help to understand the order of electrochemical gradient formation and phosphate transportation. Another important question is where the extra phosphates in *MtHA1* mutant plants come from.Application of isotope-labeled phosphate in a compartment which only AM fungal have access (Smith et al., 2003) may help verify whether the phosphate is from mycorrhizal pathway or from the improved direct pathway. Besides phosphate, the capability of AM fungi in improving other nutrients (nitrogen, sulfur, potassium, copper and zinc) raises also questions: what is the role of arbuscules in these nutrient elements transportation? Is MtHA1 needed for these transportations? Introduction of *MtHA1* mutant plants into experiments where these other nutrientsare limited or supplied to compartments where only the AM fungus has access may provide answers to these questions.

The AM fungus *F. mosseae* locally induced resistance against *A. euteiches*, but systemically induced susceptibility. Locally induced resistance is independent of full arbuscule development and JA biosynthesis, but salicylic acid seems to play a negative role. The current study provides only indications for MIR mechanisms. Thorough understanding of the AM fungus - *A. euteiches* interaction in *M. truncatula* still needs further investigations concerning e.g. the question which particular developmental stage or morphological structure during mycorrhization induces the plant immune system. Introduction of more mutants,where AM fungal colonizationis inhibited at different phases,could help to answer, if hyphopodia on the root surface, the development of the PPA, intercellular spread and/or penetration of cell walls in the root cortex and arbuscule trunk formation is responsiblefor MIR. The second important question is directed to the specific defense reactionswhich are affected during mycorrhiza

induced resistance or susceptibility? At first the hypothesis has to be tested that salicylic acid-induced responses play a crucial role. Secondly in a non-targeted approach all transcripts from the different treatments used in the current study could be sequenced and compared (RNA-seq approach). Genes which are e.g. only expressed if both, the mycorrhizal fungus and the pathogen are colonizing the roots, could be further tested by functional analysis e.g. via identification of a mutant in the transposon insertion population of Tadege et al. (2008). A third approach could come from the whole genome sequence of the pathogen (http://www.cns.fr/spip/-Aphanomyces-euteiches-.html). After identification of genes in charge of pathogenicity using proteomics or transcriptomics,the corresponding plant defense gene might be identified. The expression of such particular defense genes mostly coding for factors of signaling pathways could be modified during mycorrhiza.

6 Summary

Arbuscular mycorrhizas (AM) are wide spread mutualistic symbioses formed between fungi of the monophylum Glomeromycota and more than 80% of terrestrial plants. The central character of AM is the exchange of nutrients between partners thereby improvingphosphateacquisition of plants. In frame of this nutrient exchange, phosphate is transported across the plant periarbuscular membrane, which surrounds intracellular fungal arbuscules. This transport is driven by an electrochemical gradient build up by a proton pumping H^+-ATPase.

In frame of the current Ph.D.thesis, the role of the proton pump-encoding gene,*MtHA1*,for AM symbiotic functioningwas investigated in the model plant *Medicago truncatula*. This was achieved by analyzing a mutant where the transposon *Tnt1* is inserted in exon 8 of *MtHA1*. This insertion results in a shortened transcript which can be only translated into a protein missing an important domain for its function. In *MtHA1 exon8::Tnt1*, the AM fungus *Funneliformis mosseae*can only develop truncated arbuscules without forming typical hyphal branches. This different arbuscule morphology was mirrored by expression patterns of genes for proteins which are located in the arbuscule trunk or in the hyphal branches. Overall colonization of the root cortex decreased at late time points of the interaction and levels of mycorrhizal phosphate uptake and plant growth promotion was reduced compared to the corresponding wild type *MtHA1* plants. That the phenotype is based on the mutation in *MtHA1* could be confirmed by the dosage effect in heterozygous plants where a transposon is inserted in exon 7 of one allele of the gene. Arbuscule morphology and overall colonization was similarly affected in interactions with the rapidly colonizing AM fungus *Rhizophagus irregularis* and also with *Gigaspora rosea* forming a *Paris*-type AM. At high phosphate fertilization levels, however, colonization was already low in the *MtHA1* wild type and differences could be only observed for the morphology of the arbuscules. The root nodule symbiosis of legumes shows numerous similar molecular mechanismswith the AM symbiosis. The mutation in*MtHA1* did, however, not influence the formation of nodules and their function in improved nitrogen acquisition of the plant.

In addition to improved nutrient acquisition, AM fungal colonization can increase the resistance of roots against pathogens. The possible mechanisms for this mycorrhiza-induced resistance (MIR) were analyzed in the pathosystem *M. truncatula-Aphanomyces euteiches*. At first, it could be shown that MIR is independent of arbuscule morphology and colonization

level. *F. mosseae* was able to similarly reduce pathogen infection in the mutant *MtHA1 exon8::Tnt1*as in the corresponding wild type. Compared with pot cultures, infection of *A. euteiches* was ten times less in a split root system. This correlated with a highly increased expression of genes encoding pathogenesis-related protein and enzymes for flavonoids biosynthesis in this system. Colonization of *F. mosseae* diminished the high gene expression levels especially of*ICS*coding for isochorismate synthase, an enzyme involved in salicylic acid biosynthesis. Thisdecreased expressionwas accompanied byhigher susceptibility of the plants for the pathogen. In pot culture, which is in a different physiological stage, basal defense gene expression was low resulting in a higher pathogen infection which was reduced by the presence of the mycorrhizal fungus. This MIR could be based on reduced salicylic acid production which can lead to higher activity of jasmonate/ethylene regulated defense responses. Soluble sugar content was also analyzed, but did not show any differences. The hypothesis of competition for carbohydrates in the root must be therefore rejected for the present pathosystem.

In summary this work shows that *MtHA1*function plays a critical role in the formation and function of arbuscules. Expression of the mutated gene results in reduced formation of arbuscule branches. This in turn negatively influences mycorrhizal phosphate uptake, plant growth promotion and overall mycorrhizal colonization of the roots. MIR against *A. euteiches* is however not affected. The different physiological stages of pot culture and split root system make a comparison difficult. Gene expression analyses, however,indicate that different mechanisms underlay local and systemic interactions between the mycorrhizal fungus and the root pathogen.

7 Zusammenfassung

Arbuskuläre Mykorrhizas (AM) sind weit verbreitete mutualistische Symbiosen zwischen Pilzen des Monophylum Glomeromycota und mehr als 80% aller terrestrischen Pflanzen. Zentrale Eigenschaft der AM ist der Austausch von Nährstoffen zwischen den Partnern, der zur verbesserten Phosphataufnahme der Pflanzen führt. Im Rahmen dieses Austauschs wird Phosphat über die pflanzliche periarbuskuläre Membran transportiert. Diese Membran umgibt das intrazelluläre pilzliche Arbuskel. Der Transport wird durch einen elektrochemischen Gradienten getrieben, den eine Protonen-pumpenden H^+-ATPase aufbaut.

Im Rahmen der vorliegenden Doktorarbeit wurde die Rolle des Gens *MtHA1* für die AM Symbiose in der Modellpflanze *Medicago truncatula* untersucht. Dieses Gen kodiert für eine Protonen-pumpende H^+-ATPase. Dazu wurde eine Mutante untersucht, bei der das Transposon *Tnt1* im Exon 8 von *MtHA1* inseriert ist. Diese Insertion führt zu einem verkürzten Transkript, das nur zu einem ein Protein translatiert werden kann, dem eine wichtige Domäne für seine Funktion fehlt. In *MtHA1 exon8::Tnt1* Pflanzen kann der AM Pilz *Funneliformis mosseae* nur den Arbuskelstamm entwickeln, ohne die typischen Verzweigungen zu bilden. Diese unterschiedliche Arbuskelmorphologie spiegelte sich in der Expression von Genen wider, deren kodierten Proteine entweder im Arbuskelstamm oder in den Hyphenverzweigungen lokalisiert sind. Die gesamte Besiedelung der Wurzelrinde nahm zu späteren Zeitpunkten der Wechselwirkung ab und Phosphataufnahme über die Mykorrhiza und Wachstumsförderung war geringer als bei den entsprechenden *MtHA1* Wildtyp-Pflanzen. Dass dieser Phänotyp tatsächlich durch die Mutation in *MtHA1* begründet ist, konnte durch einen Dosiseffekt in heterozygoten Pflanzen bestätigt werden, bei denen das Transposon im Exon 7 von einem Allel des Gens inseriert ist. Geringere Besiedelung und veränderte Arbuskelmorphologie in *MtHA1 exon8::Tnt1* Pflanzen konnten auch mit den AM Pilzen *Rhizophagus irregularis*, der sich in den Wurzeln relativ schnell ausbreitet, und *Gigaspora rosea*, der eine AM des *Paris*-Typs bildet, beobachtet werden. Bei hoher Phosphatdüngung aber war schon die Besiedelung von *MtHA1* Wildtyp-Pflanzen so gering, dass sich Unterschiede nur noch bei der Morphologie der Arbuskel zeigten. Die Knöllchensymbiose der Leguminosen weist in ihren mechanistischen Grundlagen viele Gemeinsamkeiten mit der AM Symbiose auf. Die Mutation von *MtHA1* beeinträchtigte aber weder die Bildung der Knöllchen noch ihre Funktion bei der verbesserten Stickstoffaufnahme der Pflanzen.

Zusätzlich zur verbesserten Nährstoffaufnahme kann die Besiedelung mit AM Pilzen auch die Resistenz von Wurzeln gegen Pathogene steigern. Die möglichen Mechanismen dieser Mykorrhiza-induzierten Resistenz (MIR) wurden im Pathosystem *M. truncatula* - *Aphanomyces euteiches* untersucht. Zunächst konnte gezeigt werden, dass MIR unabhängig ist von Arbuskel-Morphologie und von Besiedelungsstärke. *F. mosseae* war in gleichem Maße in der Lage, den Pathogenbefall in *MtHA1 exon8::Tnt1* Pflanzen wie im Wildtyp zu reduzieren. Im Vergleich zu Topfkulturen war die *A. euteiches* Infektion von geteilten Wurzelsystemen zehnfach geringer. Dies korrelierte mit einer wesentlich verstärkten Expression von Genen, die für ‚Pathogenesis-Related' Proteine und für Enzyme der Flavonoid-Biosynthese kodieren. *F. mosseae* Besiedelung führte zu einer Reduzierung dieser verstärkten Expression. Das zeigte sich vor Allem beim Gen *ICS*, das für die Isochorismatsynthase kodiert, ein Enzym in der Salizylsäure-Biosynthese. Diese reduzierte Expression ging mit einer höheren Empfindlichkeit der Pflanzen für das Pathogen einher. In Topfkulturen, in denen sich die Wurzeln in einem anderen physiologischen Grundzustand befanden, war die basale Verteidigungsgenexpression gering, was in einer verstärkten Pathogeninfektion im Vergleich zu den geteilten Wurzelsystemen resultierte. Diese hohe Infektionsstärke wurde dann durch die Anwesenheit des Mykorrhizapilzes reduziert. Diese MIR könnte in der verminderten Bildung von Salizylsäure begründet sein, was zu einer erhöhten Aktivität von Jasmonat/Ethylen-gesteuerten Verteidigungsreaktionen führen kann. Der Gehalt an löslichen Zuckern wurde auch untersucht, aber Unterschiede konnten nicht beobachtet werden. Deshalb muss die Hypothese der Konkurrenz um Kohlenhydrate in der Wurzel in dem vorliegenden Pathosystem abgelehnt werden.

Die vorliegende Arbeit zeigt, dass *MtHA1* eine wichtige Rolle bei der Bildung und Funktion von Arbuskeln spielt. Die Expression des mutierten Gens führt zu stark verminderter Bildung der Arbuskelverzweigungen. Das beeinträchtigt schließlich Phosphataufnahme und die Wachstumsförderung durch die Mykorrhiza, sowie die gesamte pilzliche Besiedelung der Wurzel, aber nicht die Mykorrhiza-induzierte Resistenz gegen *A. euteiches*. Die verschiedenen physiologischen Zustände von Topfkulturen und geteilten Wurzelsystemen machen einen Vergleich schwierig. Die Genexpressionsanalysen legen aber nahe, dass lokale und systemische Wechselwirkungen zwischen Mykorrhizapilz und Wurzelpathogen durch unterschiedliche Mechanismen gesteuert sind.

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